

The Party's dead, but the party goes on

The Soviet Academy of Sciences was replaced last week by a Russian academy, which want a prolongation of the past. But the future of the academy will hang on what funds, if any, the Russian government can afford.

Moscow

THE Soviet Academy of Sciences went out of business on Tuesday last week, when its president, Guri Marchuk, delivered with unaccustomed emotion the "last speech of the last president of the Soviet Academy".

Marchuk was addressing more than 1,000 people assembled in the great hall of Moscow State University not so much to listen to his account of the past as to draft the charter of the new Russian Academy of Sciences which Mr Boris Yeltsin, the president of Russia, has decreed will succeed the Soviet academy.

His audience was drawn from the 250 members of the old academy (the 'academicians'), its corresponding members, the 150 people elected to an unofficial Russian Academy of Sciences before Yeltsin made his play to take over the bigger fish and representatives of the old academy's research institutes, elected to have a say at the meeting. What the last group said in public was depressingly syndicalist.

Marchuk, by uncanny coincidence, was speaking just a day after the presidents of Russia, the Ukraine and Byelorussia had consigned the Soviet Union itself to limbo by declaring at Minsk that they will in future be members of a commonwealth of their own (and not Mr Mikhail Gorbachev's) design. Perhaps for the first time in six years (Marchuk has been in office for a month more than Gorbachev), the two men were in the same boat, confronted by the reclamation of the instruments of their power by lower orders in the hierarchy.

This is how Marchuk, a cautious and uncommunicative but, only partly for those reasons, an unpopular president of the Soviet academy, described last Tuesday's proceedings: "We have gathered not only to witness, but to participate in, a performance; many of you will share my opinion that it is not just a performance, but a tragedy".

The tragedy? The dismemberment of Soviet science. The performance? The formal enactment of the fashion for "democratization". But "scientific truth cannot be found by voting, so that the search for it must be in some sense undemocratic". Marchuk offered Lysenko as typifying the "people's academicians" whose emergence he feared.

The Soviet academy, Marchuk said,

may have made mistakes, but he forecast that the new Russian academy will be a battleground for the "three major forces" that have fought over the corpse of the old academy. First, there are the gradualists such as himself. "Unfortunately, we lacked insight, skill and strength of mind. We were too dependent on those in power; we trusted their good intentions and their understanding of national problems. Posterity will justly accuse us, and will be right."

Second, there are the radicals (with the "ideological support of the press"). "They probably sincerely believe" that they can recreate Western science in Russia, but "that is a thistle that best grows on ruins, and will not yield crops". They will come to be recognized as the "destroyers of our country's science".

And the third force? The silent majority. "I appeal to you", Marchuk declared, to appreciate where "the processes started with your often passive participation" are leading, but "they are not yet irreversible". And will the Russian successor academy be able to overcome the "inevitable disappointments and losses" that lie ahead? "I believe we shall."

Marchuk, when he came to sit down, appeared as startled as his audience by what must have been the first full-throated affectionate applause he had ever earned.

It is a curious situation, as Marchuk observed. Yeltsin has decreed that the academy and its possessions on Russian soil now constitute the Russian academy, but he has not yet decreed a budget for 1992. Before that can be done, there has to be a charter. Last week's three-day meeting was an elaborate process of consultation to that end. The charter, which will be provisional for 1992, may be amended later by a mechanism not yet defined (but over which the Russian parliament will have a final say). A second three-day meeting, due to end today (19 December) will, among other things, elect a president of the new academy.

The academy's first draft, already full of democratization, is likely to be even fuller after last week. Institute directors are to be elected, and for no more than two terms. Scientific councils at research institutes will also be elected.

Research institutes will have individual charters, defining their objectives and the

means by which these may be achieved. The administrative autonomy that research institutes already enjoy to sell products and even capital equipment or land will be confirmed, as will be their freedom to decide on their researchers' salaries. That many at last week's meeting referred to the academy's institutes as "collectives" is natural enough.

Last week, the radicals were also asking for the Moon. One passionate speaker asked that the nascent academy should demand of the Russian government that the budgets for 1992 and 1993 should be identical with that for 1990, and that there should be no enforced staff reductions. The proposal was considered unrealistic in the circumstances.

The previous weekend's momentous meeting at Minsk and the inevitable consequences of the impending economic reforms (now promised for 2 January) seemed irrelevant. Yet people privately acknowledge that the pace of price inflation will then be so great as to make a nonsense of any budget, and that the government of Russia, in any case, may have no money.

Boris G. Saltikov, the able minister of science in the Russian government (said by some to be too intelligent to survive) had already spelled it out. "If you have enough money, you have freedom." He promised to provide "a certain minimum" for next year, but warned that the final budget will depend on how well inflation is contained and that "the situation is almost beyond control". In any case, 1992 will be a "period of transition", during which there will have to be a "deep restructuring of science and of the academy". Beyond that, he foresees that Russia will support only the infrastructure of research.

The unreality of last week was illustrated by the issues mentioned but hardly discussed. Should the academy continue to administer directly what it claims to be the most important slice of basic research in Russia? Marchuk is united with the radicals in the belief that separation would be disastrous. Should the academy continue to distance itself from the universities? With stalinism, the case for keeping young people out of reach of free-thinking researchers has also been buried, but the new academy is no more inclined than the old to help the universities. But Saltikov



may have other ideas.

And what on earth is to happen to industrial research, hitherto mostly the responsibility of research institutes managed by production ministries now swept away? Until industrial plants are privatized, and are able to recruit research groups of their own, Russia's applied research laboratories will have to survive on whatever crumbs of outside contracts they can win in competition with the ever-hungry academy institutes. But the academicians seem indifferent to their fates, as they are to those of the Soviet academies of medicine and agriculture.

And, in any case, can Soviet science in the large survive? Last week's meeting was predicated on the assumption that a democratized academy will continue intact. (At one point in a tortuous debate, the verb "to liberalize" was struck out in favour of "to democratize".) Only Academician L. V. Keldysh, director of the Lebedev Institute, spelled out what disasters may lie ahead if economic reform fails, or is halted by discontent.

But there is general anxiety among researchers about the rate at which able colleagues are leaving for overseas. One amendment to a draft text that would have required the academy to "study and analyse" the causes of emigration was voted down in the belief that the academy should be doing something instead.

Two needs stand out most clearly: there needs to be an office in Moscow (and perhaps in Kiev and Minsk) at which researchers can find out from Western colleagues what help is available from elsewhere, and the Russian academy and government need to make it legally possible for researchers to receive research grants directly, and to spend the money as they choose. The first should be feasible with Western help, but where democratization has not yet gone so far as to allow Russians to live wherever in Russia they choose, the second seems more distant.

Meanwhile, there are some signs of initiative and good cheer. The front runner in the election of a new academy president is Academician Ye. P. Velikhov, director of the Kurchatov Institute with a liberal reputation. And the ingenious director of the International Baikal Research Centre and the Limnological Institute at Irkutsk, Dr Mikhail Grachev, is planning to bottle Baikal water and to sell it at \$2 a litre as the purest natural water in the world.

John Maddox

Nature index issue

This unusual weekly issue is *Nature's* first attempt to produce its elaborate annual index within the year to which it refers. It is hoped that readers will not too seriously miss the regular features omitted to make room for the index. *Nature* will next appear on 2 January 1992. □

DNA fingerprinting discord

Washington

THE forensic technique of DNA fingerprinting is again at the centre of a controversy with the publication in tomorrow's issue of *Science* of an article casting doubt on customary uses of the technique.

The authors of the paper, population geneticists Richard Lewontin from the Museum of Comparative Zoology at Harvard University and Daniel Hartl of Washington University School of Medicine, are neither new to nor shy of controversy. Both have testified in recent court cases against the validity of DNA fingerprinting.

But they are offended that an official of the US Department of Justice asked them to reconsider publication and that the editors of *Science* have decided to publish a counterattack in tomorrow's issue.

DNA fingerprinting relies on knowing of several short stretches (genetic alleles) of DNA that may differ from one person to another without physiological consequences. In a courtroom, if these pieces of a suspect's DNA match samples found in connection with a crime, a suspect who would otherwise have gone free may be convicted.

The usefulness of the technique rests on reliably identifying several alleles in a DNA sample and proving that this combination is unlikely to be found in anybody else. Lewontin and Hartl challenge the assumption, central to the second task, that the probability of a match between two unrelated persons is the product of the chances that one has inherited each of the several alleles examined. Probabilities as low as one-in-a-trillion have been quoted.

Lewontin and Hartl argue that simply because a certain allele appears in only, say, one in 1,000 caucasian men, the chance of a match in all subgroups of caucasian men is not necessarily that low. The same allele might be carried by every member of a suspect's family, for example.

So, Lewontin and Hartl say, if a crime and its suspect come from a small homogeneous community, the chance that a certain allele occurs should be based on data from that subgroup alone. But such data do not generally exist, so it is impossible to calculate the likelihood of misdiagnosis. They conclude that DNA fingerprinting, at least as commonly practised, should be inadmissible evidence.

Science had accepted the paper and set it in type when, in early October, Hartl was telephoned by James Wooley, an assistant US Attorney in the Department of Justice's Organized Crime Strike Force Division in Cleveland, Ohio. Accounts

of the conversation differ. Wooley says he expressed surprise that Hartl was going to publish such a paper and that the rest of the conversation concerned the paper's scientific merits. Hartl says Wooley warned him of the "political consequences" of publishing and asked him to reconsider because of the possibly disastrous consequences for future DNA fingerprint-based prosecutions.

In a letter dated 16 October to Wooley, Lewontin accused him of a "very serious breach of ethics", saying that a request by an official in the Department of Justice Criminal Division Strike Force of a private citizen to act against his inclinations amounts to "intimidation".

Wooley, however, defends his approach to Hartl as a necessary part of forensic science, in which law and science are unavoidably mixed. "I've got just as much right to talk about the science as the scientist," he says.

Meanwhile, Daniel Koshland, editor of *Science*, had heard that news of the paper had triggered controversy at the International Congress of Human Genetics meeting in Washington, DC, and "asked to see it again". Deciding that the "discussion part went beyond the results part", he asked Lewontin to "tone it down", but Lewontin refused, threatening to accuse *Science* publicly of trying to suppress the paper.

Eventually Hartl and Lewontin agreed on some changes, but *Science* has also decided to publish a rebuttal by Ranajit Chakraborty, a University of Texas population geneticist and a frequent expert witness for DNA fingerprinting, and Kenneth Kidd, a Yale geneticist.

The rebuttal was recommended by C. Thomas Caskey, a geneticist at Baylor College of Medicine and a member of *Science's* board of reviewing editors; Caskey is a prominent supporter of DNA fingerprinting who licenses his techniques to Cellmark Diagnostics, one of the largest DNA fingerprinting companies.

He says he was concerned that "publishing defence testimony in a scientific journal" gives it such weight that courts might reopen, perhaps to overturn convictions obtained on the basis of DNA evidence.

Later this week, a National Academy of Science panel on DNA fingerprinting will meet for what is expected to be the last time before its long-awaited report on the subject. A chapter of the report will address the population issues raised by Lewontin and Hartl, based on an earlier draft of the Lewontin and Hartl paper.

Christopher Anderson

Electronic arts imitate life

Andreas G. Andreou

THE silicon neuron described by M. Mahowald and R. Douglas on page 515 of this issue¹ is a 'neuromime' which they have built using state-of-the-art microchip techniques, a silicon circuit that mimics the electrical behaviour of real nerve cells. To unearth an appropriate quote from an editorial² by John W. Moore over 30 years ago, the authors report on "an interesting, informative and perhaps provocative example of the wedding of electronic arts and concepts" to one of the life sciences.

Realistic model

Mahowald and Douglas are scientists who have mastered the art of designing analog microchips using very-large-scale-integrated (VLSI) circuit technology, which they combine with neurophysiological principles to implement a realistic model of cortical neurons. This is the same technology³ that is used to build the processor in the personal computer and the microcontrollers that fine-tune the performance of automotive engines. But whereas the latter systems use digital signals (which have only two states, the on state and the off state) and operate over discrete time intervals, analog⁴ VLSI silicon devices use signals that are graded and continuous in time. The power of VLSI technology lies in its ability to realize complex information processing in a very small volume, with minimal power dissipation and at low cost.

The silicon neuron is an attempt to harness the power of analog VLSI technology in a novel and exciting way to mimic the function of neurons and neural networks. The reported model of the neuron employs four active channels that have simplified dynamics. Real active channels are ion-carrying pores in the cell membrane that are instrumental in the electrical function of the cell. The silicon counterpart devised by Mahowald and Douglas uses a small number of electronic components native to the underlying technology, operates in continuous time and consumes minute amounts of energy. Thus many silicon neurons can be integrated and interconnected on a piece of silicon no larger than a thumbnail.

Several stereotypical neuronal properties are faithfully replicated by the silicon neuromime. When it is excited by a constant electrical current at the place that corresponds to the nerve cell body, the interactions between the active channel currents result in spiking behaviour reminiscent of action potentials recorded from living cells. The neuromime also

shows adaptation, an important property of biological neurons. When stimulated, a neuron initially fires a rapid burst of action potentials, but after a while the firing rate tails off. This is why a loud noise is annoying at first but less so with time.

The obvious application of these neuron models is in improving our understanding of brain function⁵. This is a synthetic approach to modelling the nervous system which until now has been studied using software simulations running on massively parallel digital supercomputers. For example, simulation results have been reported recently for dynamic models of the horizontal cell network⁶ in the retina, a part of the brain that is very well studied. These are simulations of realistic Hodgkin-Huxley neuron models, much like the silicon neuromime reported by Mahowald and Douglas, each having seven different ionic channels with parameters obtained from neurophysiological studies.

Simulations of a few hundred thousand horizontal nerve cells take a few minutes of supercomputer time when the stimulus has a duration of a few seconds. Similar simulations take half a day on a state-of-the-art engineering workstation. It should be noted though that horizontal neurons do not spike and that their response is analog. Networks of higher complexity, with feedback connections and a larger number of spiking neurons, could be troublesome because they are described by nonlinear differential equations that are stiff — that is, they require extremely small integration steps and a prohibitively large number of iterations.

On the other hand, an analog VLSI model, although not as accurate as digital simulations, uses the physics of the circuit elements to capture the dynamics of the problem. Therefore, we simply power up the circuit and observe the changes of voltages and currents to obtain the simulation results. This physical model has noise, irregularities and nonlinearities like any real system, including the neurons in the nervous system — properties that are computationally expensive to simulate on a digital computer. So analog VLSI could emerge as the technology of choice for modelling the nervous system. Large-scale analog simulation need not be restricted to biological systems, but can be extended to modelling systems in physics and other life sciences.

Engineers and computer scientists are working in the opposite direction, towards building a new generation of sys-

tems with brain-like capabilities⁷. They have the same options as the neuroscientists: software implementations on digital computers and novel hardware. For example, work in computational vision has shown that parallel analog algorithms can solve computationally demanding early-vision problems⁸. Compact integration of these analog algorithms in hardware requires circuit designs that are efficient in area and consume a manageable amount of energy. In this micro-power regime of operation, a new set of problems arises: devices are very slow, their characteristics are nonlinear and strongly temperature dependent. Furthermore, as we put more and more devices on a chip our computational primitives become less precise. Finally, these systems should be provided with appropriate input transducers to convert the actual physical stimuli of the real world into electrical signals.

Making progress

There are still several challenges. How can analog systems achieve the dynamic range that a particular problem requires? What should the signal representation be? How can one achieve the precision that a problem requires with inherently imprecise components? How does one guarantee that the results are independent of temperature? How can the transduction of optical, mechanical and chemical signals for vision, audition and olfaction be handled? By ingenious use of the silicon-device physics, innovative circuit-design principles and careful interpretation of the organizing principles in neural systems, researchers are making progress in these areas⁹.

From the point of view of computer science, concerns such as those outlined above are a reminder that computation is a physical process governed by the physics of the computational substrate. A science of computation is emerging from both biological and engineering concerns that someday may lead to large-scale information-processing systems that approach the efficiency and robustness of neural systems. □

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Immunization in the field

Roy M. Anderson

ON page 520 of this issue¹, Brochier and colleagues report the success of the first large-scale field trial of a recombinant vaccinia-rabies vaccine in immunizing populations of the red fox (*Vulpes vulpes*). The trial took place in Belgium, where (as in the rest of Western Europe) the fox is the main reservoir of the rabies virus. In humans, the disease is particularly distressing — its course is marked by severe convulsions and hydrophobia, and it is invariably fatal.

The rabies virus invades the central nervous system of its host and is typically transmitted to humans by the bite of an infected animal. The disease is virtually worldwide in distribution and its epidemiology is characterized by maintenance of the virus in different mammalian wildlife species in different regions of the world. Although there is great antigenic similarity between viral strains isolated from various hosts, a given strain appears to be highly host specific, implying that host and pathogen have coevolved in areas of endemic infection. Rabies was known to human civilization as long ago as 2300 BC, as was the fact that it could be transmitted by the bite of a 'mad' dog². Occasionally, the disease seems to have attained epidemic proportions, which may have been the result of the emergence of new, highly virulent strains of the virus.

In Europe, the main concern is fox

rabies, its transmission to domestic animals and the associated risk to humans. The current epidemic started in Poland during the Second World War and has gradually spread to most of the central and Western European countries, and eastward into the Soviet Union. Initial efforts at control were directed at a reduction in fox numbers, with the aim of reducing density below the critical level required for the persistence of the infection (thought to be around 0.4 foxes per km²). But continued culling is required to maintain an area free from rabies, with implications both for cost and for maintenance of culling when the disease is rare³.

The other, more recent approach to control has been investigation of the potential value of oral vaccination of foxes⁴. Preliminary results from a trial with live attenuated viral strains were promising⁵. But a major drawback to widescale use was the nature of the attenuated virus used in the vaccine, which retained a degree of virulence.

A few years ago, however, a recombinant vaccinia-virus vaccine (VVTG-gRAB) expressing the surface glycoprotein G of the rabies virus (ERA strain) was developed⁶. The vaccine appears to be highly efficacious in experimental animals, captive foxes and racoons, and to be safe for laboratory and domestic animals, as well as other wildlife species

likely to come into contact with inoculated baits⁷. Brochier and colleagues now report its use in a trial in a 2,200-km² region in southern Belgium, following a successful smaller trial to assess safety.

The results are impressive. In a three-phase distribution programme between November 1989 and October 1990, helicopters distributed 25,000 baits containing live VVTGgRAB vaccine and a tetracycline marker (to identify vaccine-induced immunity in the fox population). Following the final phase of distribution, vaccine-induced immunity was evident in 81 per cent of the foxes sampled. Calculations based on epidemiological theory⁸ suggest that, in an area with an average fox density of two animals per km², roughly 80 per cent would have to be immunized to block transmission (that is, to reduce the rate of production of secondary cases of infection, generated by one primary case, to below unity). The observed outcome was in accord with this prediction — the incidence of rabies declined rapidly over the trial period, and no cases of the disease in either foxes or domestic animals have been reported in the area since June 1990.

Encouraging though the results are, a number of problems must be addressed before the strategy can be widely adopted. First is the cost. The authors provide figures to show that the overall expenditure during the trial was not much more than that associated with fox culling over a similar period of time. But although mass immunization of wildlife reservoir species may indeed turn out to be a viable option in the West, in poorer countries (including those of Europe) treatment of other infectious diseases is likely to have higher priority.

Second, there is the interpretation of the trial results. The design of the trial did not include a comparable area in which baits were not distributed, perhaps understandably, given the difficulty of matching two large areas with similar habitats and fox densities. However, the incidence of rabies is typically cyclical in fox populations, with an interepidemic period of 3–6 years depending on fox density⁸. If the trial was carried out in an interepidemic period, the incidence of rabies would be expected to be much lower than in previous years. So a period of observation covering both epidemic and interepidemic years is really required to assess fully the effect of the strategy.

A further need is to improve upon the low levels of immunization attained in juvenile animals (49 per cent, against the 80 per cent achieved in adults). Juveniles can play an important part in rabies transmission: they may represent up to 70 per cent of the total population in

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May and June⁹, and they disperse widely from the parental habitat to seek empty territories.

The final issue is the overall level of vaccination achieved in the trial. In areas of low to moderate fox density, simple theory suggests that 80 per cent coverage is sufficient. If fox density was of the order of 4 per km² (not unusual in some suburban areas in Europe¹⁰), then a similar calculation produces a figure of 90 per cent to block transmission. It was estimated that the typical density of foxes before the trial was 2 per km². But given that some hunting and gassing took place in the preceding years, the habitat might be able to support much higher densities. If vaccination replaces culling, then fox density is likely to rise, as a consequence both of the decreased incidence of rabies (the disease has a considerable effect on fox abundance) and of the cessation of culling. So unless culling is continued, a level of vaccine uptake of over 80 per cent may be required to prevent the re-establishment of the virus.

Nonetheless, the trial results show that mass vaccination may be an alternative (in some countries) to the continual culling of foxes. Aside from long-term control, the availability of a safe and

effective vaccine for wildlife raises many other possibilities, such as the control of local outbreaks of rabies in rare mammalian species (one example is the African wild dog, *Lycaon pictus*, in certain East African game reserves, where rabies is threatening the survival of the species). Above all, perhaps, the development of a method of disease control not involving the slaughter of wildlife would be only too welcome in the conservation-conscious social climate of the 1990s. □

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The mutant protein responds

Jeffrey J. Wine

CYSTIC FIBROSIS results from mutations in a single gene that codes for the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel requiring both cAMP-dependent phosphorylation and ATP hydrolysis to open¹. At least in some cells, most mutations that give rise to cystic fibrosis cause the CFTR protein to be misprocessed so that little of it reaches the membrane^{2,3}, providing a direct explanation for the reduced Cl[−] conductance in affected tissues⁴. But Dalemans *et al.*, on page 526 of this issue⁵, and Drumm *et al.*, in this week's *Science*⁶, now report that mutant CFTR *does* reach the membrane when expressed in fibroblasts⁵ or oocytes⁶, and that, against expectations, cells expressing mutant CFTR become responsive to stimulation. These are exciting results — they offer hope for therapies based on improving the functions of mutant CFTR, which might be important adjuncts to strategies based on replacing the defective gene or protein.

Dalemans *et al.* expressed wild-type or mutant (Δ F508) CFTR in fibroblasts. Consistent with evidence that Δ F508 protein is misprocessed^{2,3}, Δ F508 CFTR was not detected in the plasma membrane by surface labelling, but low levels

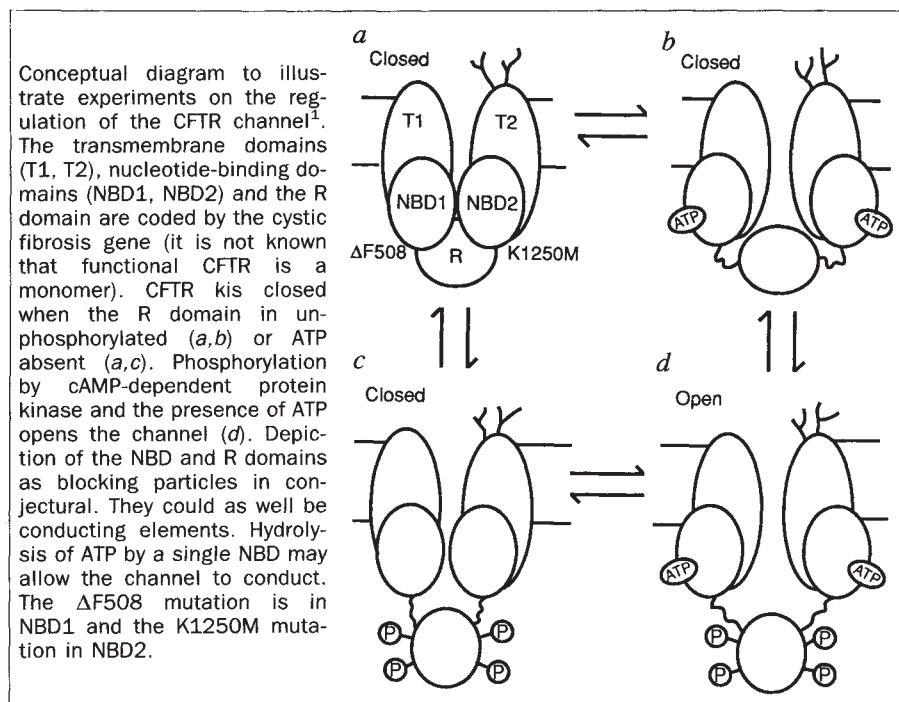
of channel proteins can produce significant physiological responses. Drumm *et al.* expressed wild-type and several mutant versions of CFTR in *Xenopus*

oocytes. On the main points, both groups agree.

In cells expressing normal or Δ F508 CFTR, but not in mock-transfected fibroblasts or uninjected oocytes, increases in anion conductances were observed following stimulation with a cocktail of forskolin, IBMX and cpt-cAMP, all of which elevate levels of cAMP (but by different means). Iodide efflux, whole-cell Cl[−] currents or single-channel activity were measured in fibroblasts, and Cl[−] currents were measured in voltage-clamped oocytes. Responses of cells expressing Δ F508-CFTR were 10–25 per cent of the magnitude of wild-type responses, and were qualitatively similar with one exception: the stimulated I[−] efflux (fibroblasts) or Cl[−] currents (oocytes) had much slower time courses and were more sustained in cells expressing Δ F508. In fibroblasts, single-channel currents attributed to Δ F508 expression appeared to be normal in most respects, but fewer active channels were observed and closed times were at least three times longer than in wild-type CFTR⁵.

Drumm *et al.* also varied IBMX levels up to 5 mM, a level required for complete inhibition of phosphodiesterase in oocytes. Although no further increases in Cl[−] current were observed in oocytes expressing CFTR, currents in cells expressing Δ F508 CFTR grew in response to increases of IBMX until they were about 60 per cent of the wild-type responses. Similar results were obtained after expressing two other disease-related CFTR mutants.

The new results^{5,6} differ from previous ones, in which forskolin and IBMX failed to stimulate anion fluxes in cells expressing recombinant Δ F508 CFTR^{3,7}.



Substantial residual function of $\Delta F508$ CFTR is also unexpected given the near total loss of sweat-duct Cl^- permeability⁴ and cAMP-mediated sweat secretion⁸ in people with cystic fibrosis. These differences can be reconciled in two ways: the earlier experiments might have used inadequate stimuli to reveal the residual function of $\Delta F508$ CFTR, or the amount of mutant CFTR that reaches the membrane may vary among cells or expression systems. So it still must be determined if normal amounts of $\Delta F508$ CFTR reach the plasma membranes of native epithelial tissues — and one answer may not apply to all tissues.

Given that some $\Delta F508$ CFTR reaches the membrane in these systems, how do the results fit into the emerging story of CFTR structure and function, exemplified by the recent work of Anderson *et al.*¹? Their evidence that ATP hydrolysis is required for regulating CFTR conductance is compelling — ATP γ S and cAMP-dependent protein kinase (PKA) supported phosphorylation but did not increase conductance; but removal of PKA followed by addition of ATP did increase conductance (see figure). Surprisingly, ATP hydrolysis was also required to increase conductance in CFTR mutants lacking most of the R domain. Residual function might be explained by proposing that $\Delta F508$ does not interfere with ATP use by the first nucleotide-binding domain (NBD1), or that ATP use by the second (NBD2) can maintain partial function. It may be relevant that the CFTR mutant K1250M, which is predicted to be unable to hydrolyse ATP in NBD2, nevertheless responds to ATP with increased activity³. (K1250M is processed normally and is not known to be associated with cystic fibrosis.) So ATP hydrolysis by either nucleotide-binding fold may be sufficient for channel opening, although with reduced efficiency.

Testing this and other hypotheses requires that mutant CFTR makes it to the membrane to be available for testing. The new results^{5,6} establish that such experiments are feasible, and so will further galvanize efforts to define the molecular basis of normal and mutant CFTR function. □

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Ice right under the Sun

Clark R. Chapman

THE apparent discovery of a polar ice cap on Mercury, the innermost of the planets at a mere 58 million kilometres from the Sun, caused consternation when it was announced at a meeting on planetary sciences last month*.

Early this century, the closest planet to the Sun seemed to be a nice place to visit. Sure, it was hot at high noon, but Mercury's back side, always hidden from the Sun, was thought to be the coldest place in the Solar System. There were moderate temperatures near the day/night boundary. Because of Mercury's elliptical orbit, the transition zone actually migrated rapidly to and fro, but near the poles, Mercury could have Earth-like temperatures.

Moreover, clouds were 'seen' drifting over the planet's surface. At least three observers, viewing the small, shimmering planet through telescopes, reported glimpsing a bright polar cap, resembling the ice caps on Mars. Even as late as the mid-1960s, several astrophysical observations indicated (although not without controversy) that Mercury had an atmosphere similar in density to the one we now know exists on Mars.

But our picture of Mercury changed radically in 1965, when radar echoes received by the 300-metre Arecibo dish in Puerto Rico demonstrated that Mercury's day is 59 Earth days long, not 88, as had previously been believed. That meant that Mercury does not keep one face to the Sun at all times on its 88-day orbit, so it has no permanently frigid back side. Also, many of the signs previously taken to indicate the existence of an atmosphere suddenly had much simpler explanations in terms of the true rotation period.

In March 1974 and on two subsequent occasions, the Mariner 10 spacecraft studied the innermost planet close up, revealing its crater-scarred surface and its unexpected magnetic field. Detailed data were obtained about its atmosphere, which turned out to be a billion times thinner than the Mars-like atmosphere under discussion in the 1950s and early 1960s. The broiling surface has remained of interest mostly to specialists, who have searched for clues to Mercury's bulk composition and who theorize about interactions between gas molecules and Mercury's surface grains in order to understand recent observations of changing spectral emission lines of sodium and potassium in the planet's tenuous atmosphere. But all thoughts of

a clement polar climate were relegated to history.

Hence everyone's surprise last month when radar astronomers, using the Jet Propulsion Laboratory's (JPL) Goldstone tracking antenna, the Very Large Array in New Mexico, and the Arecibo facility, announced that Mercury may have polar ice caps. During Mercury's relatively close approach to Earth in August, Martin Slade (JPL) and Duane Muhleman (California Institute of Technology) organized an observational assault on the hemisphere left unobserved by Mariner 10.

Mercury's north pole happened to be tipped slightly towards Earth in August, and the images reconstructed from the radar echoes showed a prominent, radar-reflective 'cap' situated precisely at the pole. The history of planetary radar astronomy has turned up anomalously bright echoes before — from Saturn's rings, the surfaces of some of Jupiter's satellites, and from the small Earth-approaching object Adonis. Some of these data have been explained in terms of the properties of water ice.

At first, ice caps on Mercury seemed as unlikely to Slade and his associates as they did to most of those at the planetary sciences meeting where the discovery was announced. But David Paige (University of California at Los Angeles) ran some simplified thermal models and concluded that Mercury's polar regions could be well below freezing, perhaps only 125 K.

Actually, Gary E. Thomas, writing in *Science* (**183**, 1197–1198; 1974) just before Mariner 10's first reconnaissance of Mercury, proposed that ice could have accumulated in the small planet's polar regions, perhaps in permanently shaded regions. Unlike the Moon, where polar ice deposits have been hypothesized, Mercury's poles are always oriented precisely 90 degrees away from the Sun–Mercury line, so there are likely to be larger regions of permanent shadow. Certainly, the sunlit polar regions photographed by Mariner 10 show no hint of exposed ice. But permanently shadowed regions are unlikely to be extensive enough to account for the broad north-polar cap observed by radar.

Slade and colleagues think it more likely that extensive ice fields could be buried several metres deep beneath Mercury's soil. Temperatures would be permanently cold there, loss of water molecules would be inhibited, and — of course — the soil would have hidden the ice from Mariner's cameras while allowing penetration of the radar waves.

*23rd Annual Meeting of the Division for Planetary Sciences of the American Astronomical Society, Palo Alto, California, 4–8 November 1991.

If Mercury truly has ice caps, where would the water be coming from? Despite Mercury's magnetic field, the solar wind occasionally reaches the surface, so hydrogen could be implanted into the surface. Mercury's abundant silicate crustal rocks contain oxygen, so there is a theoretical chance that some hydrogen and oxygen could get together and form water. More likely, the water has come from the comets and carbonaceous asteroids that have been bombarding Mercury since the planet's formation until the present day. Some water may still be outgassing and there is certainly continual replenishment from space.

Obviously, in Mercury's hot, radiative environment, the vast majority of water molecules near its surface are quickly dissociated and lost to space. But some small fraction of them may find their way to the planet's polar cold traps and diffuse downwards to subsurface ice traps. Despite Thomas's 1974 suggestion, little subsequent research on Mercury's atmospheric processes has considered the possibility of polar ice caps. Already, new calculations are underway and we may soon expect some revisionist models of Mercury's atmosphere.

Lest we get carried away, however, it

remains unproved that the reflective deposit at Mercury's north pole is due to water ice. The Magellan spacecraft's fantastic radar maps of Venus's surface have also revealed anomalously bright units that can hardly be made of ice. The coincidence of Mercury's cap with its north pole (and the hint of a similar feature near its south pole) clearly points to a temperature-sensitive substance as the culprit.

On the one hand, we can see now that our 'broiling Mercury' *gestalt* had inhibited our thinking about ice on Mercury, and ice may be shown to be perfectly reasonable once all the calculations of sources, sinks, transport processes and stability are done. On the other hand, we live on an exceptionally watery planet that happens to have a mean temperature very near the freezing point of water, so there is a possibility that ice comes too readily to our minds. Until we understand the radar reflectivity properties of all other plausible materials, we will not know for certain that there is ice right under the Sun. □

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OLIGONUCLEOTIDE DRUGS

A change of backbone

Maxim Frank-Kamenetskii

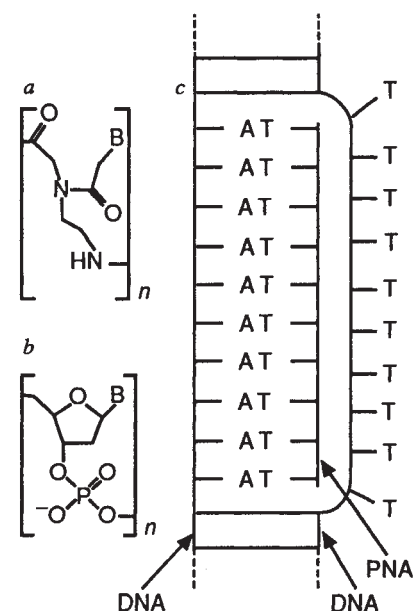
STUDY of chemically engineered analogues of natural oligonucleotides is all the rage, not least because of their potential as therapeutic agents which act by selectively blocking gene expression. The most radical departure to date from natural oligonucleotides is now reported by Peter Nielsen and colleagues (*Science* **254**, 1497–1500; 1991), who replaced the entire sugar-phosphate backbone of a stretch of DNA with a polyamide chain, preserving only the bases from the DNA prototype. Such protein–DNA chimaeric oligonucleotides form extremely stable heteroduplexes with complementary single-stranded DNA.

The promise of synthetic oligonucleotides is in their potential ability to silence particular genes at the level of messenger RNA (the antisense strategy) or that of double-stranded DNA (the triplex strategy); see *Nature* **350**, 442–443 (1991) for a brief review. They are also finding application as sequence-specific markers on both single- and double-stranded DNA. Increasing evidence has been accumulating to the effect that such 'gene-drugs' make it possible to affect gene expression in both isolated cells and entire organisms.

In genetically engineered organisms, it has proved feasible to incorporate

special constructions that yield natural RNA oligonucleotides endogenously. But for clinical application one has to look to synthetic analogues of oligonucleotides that can penetrate cell walls and be efficiently attached to intracellular DNA or RNA. In the search for such analogues, which could improve the accessory properties of oligonucleotides such as nuclease resistance and cell penetration, various groups have been working on modification of the oligonucleotide sugar-phosphate backbone. It is a tricky business, though, because the replacement of even a single atom can significantly decrease the stability of the complex between the oligonucleotide and single- or double-stranded DNA. The achievement of Nielsen *et al.* is to show that whereas small changes in the backbone may be profoundly destabilizing, big changes need not be.

Nielsen *et al.* used computer modelling to design their polyamide nucleic acid (PNA), completely replacing the sugar-phosphate backbone with a polyamide chain. The only common element between their ersatz oligonucleotide and the normal one is the base (designated as B in the figure). They then synthesized oligo-PNAs containing from six to ten thymines. When they mixed the product



a, The structure of polyamide nucleic acid (PNA), in which the sugar-phosphate backbone of DNA (b) is replaced with a polyamide chain; only the base (B) remains the same. c, Strand displacement of the T-strand of linear plasmid DNA by oligo-PNA. (From Nielsen *et al. Science* **254**, 1497–1500; 1991.)

with dA₁₀, a nucleotide string of ten adenines, duplexes formed. Quite amazingly, the melting temperature of this heteroduplex was 50 °C higher than the melting temperature of the normal DNA duplex formed between dT₁₀ (ten thymines) and dA₁₀.

The next experiment further emphasized the unprecedented stability of the PNA–DNA heteroduplex. When oligo-PNA was mixed with linear plasmid DNA carrying a dT₁₀–dA₁₀ tract, it was found that, rather than forming a triplex, oligo-PNA displaced the T-strand and formed a duplex with the A-strand (part c of figure). This was convincingly demonstrated in a series of experiments involving enzymatic and chemical probing. It remains to be seen whether oligo-PNAs with an arbitrary sequence will form such a strong complex, although it seems likely that they will.

The availability of this new sequence-specific agent with such strong affinity for single-stranded DNA (and, most probably, for RNA) seems to open a cornucopia of opportunities. Among them are synthesis of much more potent antisense oligonucleotides, of markers that bind to specific sites independently of whether they are in duplex or single-stranded form, and of artificial restriction enzymes for any sequence. But a pivotal question remains: will such strong affinity be associated with significant loss of the sequence specificity? □

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Formation and resolution of recombination intermediates by *E. coli* RecA and RuvC proteins

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The recombination of DNA molecules has been reconstituted *in vitro* using two purified enzymes from *Escherichia coli*. RecA protein catalyses homologous pairing and strand exchange reactions to form intermediate DNA structures that are acted upon by RuvC. The newly identified RuvC protein resolves the intermediates by specific endonucleolytic cleavage to produce recombinant DNA molecules.

THE formation of a nucleoprotein complex is central to the process of homologous recombination in prokaryotic organisms. Genetic recombination in *E. coli* requires RecA protein (relative molecular mass 37,842 (37.84K) which polymerizes on DNA to form a right-handed helical filament with a pitch of 95 Å (ref. 1). The DNA lies along the longitudinal axis of the filament and is stretched 1.5 times relative to the length of B-form duplex DNA. The primary function of the nucleoprotein complex is to bring two DNA molecules into close proximity, with homologous sequences in alignment ready for strand exchange and the formation of heteroduplex DNA^{2,3}.

RecA catalyses the pairing of single-stranded circular and linear duplex DNA by the formation of a triple-helical DNA intermediate⁴⁻⁷. These structures are thought to involve hydrogen-bonding of the single-strand in the major groove of the duplex, as indicated by thermal stability studies of deproteinized reaction intermediates^{5,7}. Mechanistically, the pairing of duplex molecules by RecA may be similar, with two duplexes paired in the form of a four-stranded DNA helix^{2,8,9}. Removal of RecA protein leads to the formation of a classical Holliday junction, in which two duplex molecules are linked at a single crossover point¹⁰⁻¹².

Following homologous pairing and strand exchange, recombination intermediates need to be resolved into recombinant DNA products. Resolution is thought to occur by specific endonucleolytic cleavage of the Holliday junction, but little is known about the mechanism of resolution or about the cellular enzymes that catalyse these events. Efforts to understand the process have focused on the biochemical properties of bacteriophage T4 endonuclease VII, a packaging enzyme that resolves junctions as part of its broad substrate specificity¹³⁻¹⁵, and on structural studies of synthetic DNA junctions¹⁶.

It is important to identify and characterize the bacterial enzyme that resolves recombination intermediates, and we have reported a resolution activity in cell-free extracts of *E. coli*¹⁷. Subsequent experiments showed that this activity was absent from extracts made from the recombination/repair defective mutant *ruvC*¹⁸, and provided a key to the identification of the elusive resolvase enzyme. Here we demonstrate that the 19K

RuvC protein resolves recombination intermediates made by RecA protein. Using suitable DNA substrates, the combined action of RecA and RuvC leads to the formation of recombinant DNA molecules *in vitro*. Moreover, using small synthetic DNA junctions, we show that resolution depends on the presence of homologous DNA sequences at the junction point.

RecA and RuvC mediate recombination *in vitro*

To determine whether the product of *ruvC* plays a direct role in resolution, a 1.12-kilobase (kb) *StuI-EcoRV* fragment of the *E. coli* chromosome carrying *ruvC* was cloned into pUC18 (ref. 19). The resultant plasmid, pGS762, carried *ruvC* under the control of the *lac* promoter. The 19 K RuvC protein was purified from *E. coli* strain FB800 (a *ruvA60::Tn10* derivative of JM101) carrying pGS762 (Fig. 1a).

To analyse whether RuvC can resolve recombination intermediates *in vitro*, we made use of the *E. coli* RecA protein (Fig. 1a). Using gapped circular DNA and ³²P-end-labelled linear duplex ΦX174 DNA (Fig. 2, top left), RecA catalyses homologous pairing and strand-exchange reactions to form ³²P-labelled nicked circular and ³²P-labelled gapped linear products (Fig. 2, bottom left)^{20,21}. The conversion of the substrates (Fig. 1b, lane a) into products (lane b) by RecA protein is detected by agarose gel electrophoresis (note that only ³²P-labelled DNA will be seen on the autoradiogram).

Addition of RuvC to the RecA-mediated strand exchange reaction produced a new DNA species that migrated at the position of linear dimer DNA (Fig. 1b, lanes c-i). The concentration of RuvC was critical and little product was observed at very high protein concentrations (lane i). But at the optimal concentration of RuvC (about one monomer per 80 nucleotides), a high percentage of the recombination intermediates were converted into linear dimer products (lane g). In a control that contained RuvC, but no RecA, this DNA product was not observed (lane j). Analogous reactions, using either T4 endonuclease VII (ref. 21), or a partially purified fraction from *E. coli*^{17,18}, have shown that the linear dimer is formed by resolution of the recombination intermediates made by RecA (Fig. 2, cleavage at a-c). Restriction analyses indicated a molecule equivalent to a recombinant product (with exchanged outside markers).

A time course of the resolution reaction is shown in Fig. 3. Two parallel reactions were set up, one contained RecA only and the other contained RecA and RuvC. Samples were removed at various times and the DNA products analysed by agarose gel electrophoresis. Analysis of the RecA reaction (lanes b-g) showed that after 5-15 minutes a large proportion of the ³²P-label is found as slow migrating strand exchange intermediates. At later times (30-60 min), ³²P-labelled nicked circular duplex products are formed, indicating completion of strand exchange. In the reaction containing RecA and RuvC (lanes h-m), linear dimer DNA is observed soon after initiation of the reaction (5-15 min). At these times, we also observed the formation of a small amount of ³²P-labelled nicked circular DNA.

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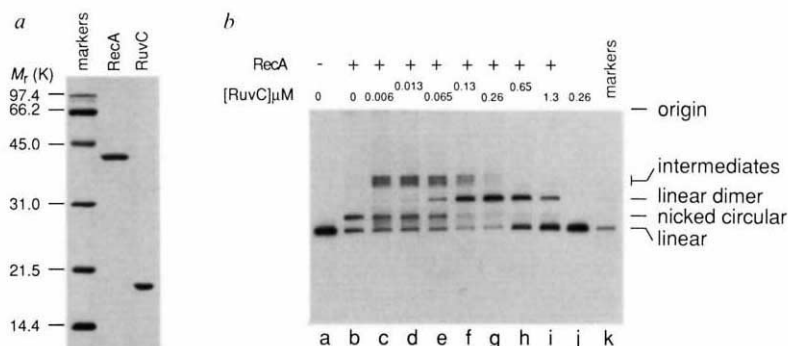
The early formation of nicked circular DNA may be due to RuvC-dependent resolution that gives rise to parental products (Fig. 2, cleavage at b-d). This result is consistent with studies with partially purified fractions¹⁷, in which there was a bias towards the recombinant product (linear dimer DNA). The bias is less than that indicated by the gel analysis, however, because the linear dimer contains two ³²P-labels compared with the single ³²P of the nicked circular product (Fig. 2).

To determine whether the bias was due to the presence of

RecA protein, recombination intermediates were deproteinized by treatment with proteinase K and SDS and purified by gel filtration chromatography. When protein-free intermediates were treated with RuvC, resolution occurred with a frequency closer to 1:1 (data not shown). The resolution reaction showed no requirement for ATP. Thus, RuvC protein resolves recombination intermediates to form recombinant products with parental and recombinant configurations of the flanking arms.

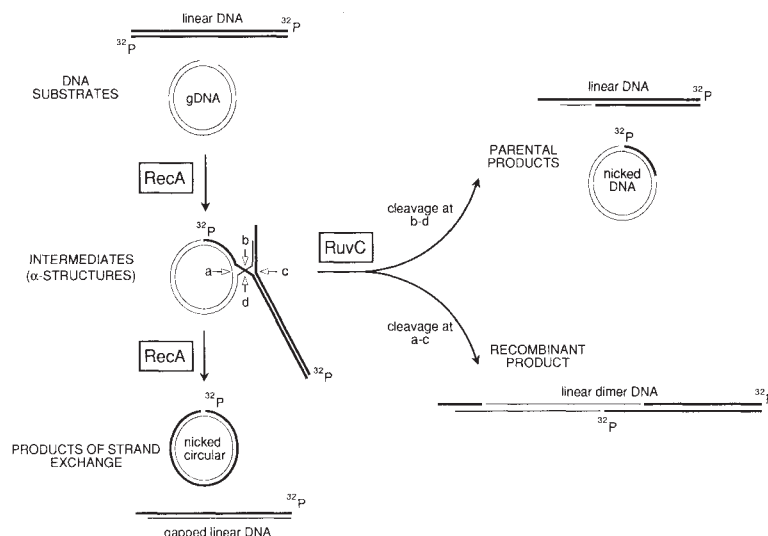
FIG. 1 The formation and resolution of recombination intermediates using purified *E. coli* proteins. *a*, RecA and RuvC proteins (0.5 µg) were subjected to SDS-PAGE on a 13.5% gel with *M_r* standards (BioRad Labs). *b*, Formation and resolution of recombination intermediates using purified RecA and RuvC. Reactions (20 µl) containing gapped circular DNA (15.6 µM), 3'-³²P-end labelled linear duplex DNA (5.2 µM) and RecA protein (7.6 µM) were incubated at 37 °C (reaction shown in Fig. 2). RuvC was added after 5 min and the incubation continued. The presence (+) or absence (-) of RecA and the concentrations of RuvC are indicated (expressed in moles of monomeric protein). Reactions were stopped after either 60 (lanes a and b) or 30 (c-j) min and deproteinized by addition of phenol, sarcosyl and EDTA (to 12.5%, 0.38% and 18.8 mM respectively). Products were analysed on a 0.7% agarose gel in Tris-acetate/EDTA buffer and ³²P-labelled DNA detected by autoradiography.

METHODS. RuvC protein was purified from *E. coli* strain FB800 (*ruvA::Tn10* derivative of JM101) carrying pGS762 *ruvC* (ref. 19). Six litres of cells were grown in Luria broth (with 100 µg ml⁻¹ carbenicillin) to an absorbance at 650 nm of 0.5, and induced by addition of 1 mM isopropyl-β-D-thiogalactoside followed by 4 h growth. Cells (20 g) were collected by centrifugation, resuspended in 60 ml 100 mM Tris-HCl, pH 8.0, 2 mM EDTA, 5% (v/v) glycerol and stored at -70 °C. When required, the cell paste was thawed and all further procedures carried out at 4 °C. Dithiothreitol was added to 1 mM and extracts prepared by sonication (4 × 15 s pulses using an MSE Soniprep 150 at maximum amplitude). Salt (0.2 volumes 5 M NaCl) and detergents (0.01 volumes 10% Triton X-100 and 0.1 volumes 4% sodium deoxycholate) were added to avoid losses by DNA and membrane binding, and high-speed supernatants prepared by centrifugation at 42 × 10³ r.p.m. for 60 min in a Beckman 60 Ti rotor. The supernatant (90 ml) was dialysed against R buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol) containing 0.1 M KCl for 3 h, and soluble proteins (~200 mg) loaded onto a 20 ml phosphocellulose column equilibrated with the same buffer (flow rate 30 ml per hour). Bound proteins were eluted with a 300-ml linear gradient of KCl (0.1–1.0 M) in R buffer. Fractions containing the 19K RuvC protein, which eluted at around 0.45 M KCl (as identified by SDS-PAGE), were pooled (22 ml, 0.16 mg ml⁻¹), dialysed against R buffer for 3 h, and applied to a 5 ml single-stranded DNA cellulose column (flow rate, 20 ml per hour). Bound proteins were eluted with an 80-ml linear gradient of KCl (0–0.5 M) in R buffer, and fractions containing RuvC (which



eluted at about 0.3 M KCl) were identified by SDS-PAGE (9 ml, 72 µg ml⁻¹). At this stage the fractions could be stored overnight at -20 °C after addition of glycerol to 45% (v/v). After dialysis for 3 h against R buffer containing 0.1 M KCl, protein was applied to a 1.5-ml reactive blue 4-agarose (Sigma) column (flow rate 20 ml per hour). A 24-ml gradient of KCl (0.1–2.0 M) in R buffer was used for elution, and peak fractions containing RuvC (eluting at about 1.0 M KCl) were dialysed against R buffer containing 45% glycerol and 50 mM KCl and stored in aliquots at -70 °C (1.6 ml, 0.25 mg ml⁻¹). The identity of RuvC protein was confirmed by sequencing the first 24 amino-acid residues. During the course of purification, fractions were also assayed for the cleavage of (1) synthetic junctions, and (2) recombination intermediates made by RecA. Resolution activity co-purified with the 19K RuvC protein. The concentration of RuvC was determined using the BioRad protein assay (bovine serum albumin as standard). RecA was purified as described⁴⁴. Gapped circular and 3'-³²P-labelled *Pst*I-linearized duplex DNA were prepared from ΦX174 DNA as described^{17,21}. The gapped circular DNA contained a 162-nucleotide gap in the minus strand defined by the *Pst*I and *Av*II restriction sites. DNA concentrations are expressed in moles of nucleotide residues. Strand exchange/resolution reactions were carried out in 50 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, 1 mM dithiothreitol and 2 mM ATP. An ATP-regenerating system was included (10 mM phosphocreatine and 5 units per ml phosphocreatine kinase). The linear dimer and linear monomer size markers (lane k) were produced by ligation of linearized ΦX174 DNA

FIG. 2 Model system used in the experiments. The substrates are gapped circular DNA and ³²P-labelled linear duplex DNA (top left). RecA protein catalyses homologous pairing and strand exchange to form intermediates (α structures) and then products (³²P-labelled nicked circular and ³²P-labelled gapped linear DNA; bottom left). Resolution of the intermediates gives rise to ³²P-labelled linear dimer DNA (cleavage at a-c) and ³²P-labelled nicked circular and ³²P-labelled gapped linear DNA (cleavage at b-d). In genetic terms these are equivalent to the formation of recombinant products with either recombinant or parental configurations of the flanking arms respectively. 3'-termini used for ³²P-labelling are indicated. The drawings are schematic and do not take into account the possibility of three- or four-stranded helical complexes. These may be present at the time of resolution.



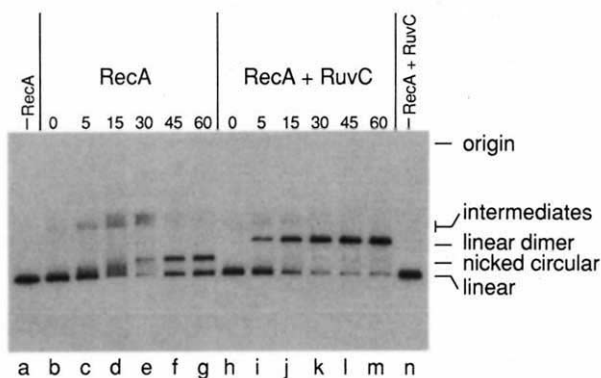


FIG. 3 Time course of *in vitro* recombination reaction. Two reactions (80 μ l) containing gDNA (15.6 μ M), 3'- 32 P-labelled linear duplex DNA (5.2 μ M) and RecA protein (7.6 μ M) were incubated at 37 $^{\circ}$ C. After 5 min, RuvC protein (0.26 μ M) was added to one reaction and this time was designated $t=0$. Incubation was continued and at the indicated times (in min) 10 μ l samples were withdrawn from both reactions, stopped and deproteinized. Controls (lanes a and n) and reaction conditions were as described for Fig. 1. 32 P-labelled DNA was visualized by autoradiography following electrophoresis through 0.7% agarose.

Specificity of RuvC

The RecA-mediated strand exchange reaction between unlabelled single-stranded circular and 32 P-labelled linear duplex DNA (Fig. 4, lane a)²², leads to the formation of 32 P-nicked circular and 32 P-linear single-stranded DNA (lane c). To determine whether intermediates of this reaction could be resolved by RuvC, three- and four-stranded reactions were set up in parallel and supplemented with RuvC. Figure 4 shows that whereas RuvC resolves four-stranded DNA intermediates (lanes k-n), its only effect on the three-stranded reaction (lanes c-h) is an inhibition of RecA-mediated strand exchange at the highest RuvC concentration (lane h). These results may indicate a specificity for four-stranded intermediates, although we cannot exclude the possibility that resolution might be observed under different experimental conditions.

The data shown in Figs 1, 3 and 4 indicate that RuvC is specific for junctions and has little or no degradative effect on the DNA substrates (gapped circular DNA, linear duplex, or single-stranded DNA). Moreover, under standard reaction conditions (Fig. 1 legend), we found that single-stranded DNA from Φ X174 (30 μ M) was stable when incubated alone with RuvC (0.65 μ M) for 30 min (data not shown).

Resolution of synthetic junctions

In the reactions described, RecA generates DNA intermediates that serve as substrates for RuvC. To dissect the reaction and look at resolution in the absence of RecA protein, we constructed a synthetic junction (J12) that was 50 nucleotides in length. The structure, shown in Fig. 5a, contains a homologous core (region J) of 12 base pairs (bp) flanked by heterologous sequences (regions A, B, C and D). In the presence of EDTA, RuvC binds 32 P-labelled J12 to form a single protein-DNA complex (Fig. 5b, lanes a-e). Under the same conditions, binding to linear duplex DNA was not detected by the band-shift assay (lanes g-j).

In the presence of Mg^{2+} , J12 was resolved by RuvC to form products that comigrated with a linear duplex marker, as detected by PAGE (Fig. 5c, lanes b-e). Cleavage occurred as described¹⁸ by the introduction of symmetrically related nicks into two strands at the sequence 5'-TGT↓CCT-3' (data not shown). Cleavage of a linear duplex has not been observed, either by neutral (Fig. 5c, lane g) or denaturing PAGE (data not shown).

Homology requirement

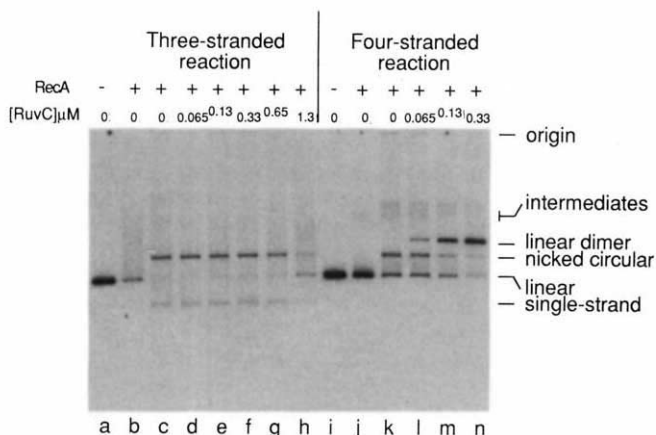
To determine whether RuvC requires homologous DNA sequences for resolution, we constructed two new synthetic junctions in which the 12-bp homologous core of J12 was reduced to 6 (J6), and to 3 (J3) bp. We also used a junction that lacked homology (J0) and a three-armed Y-structure.

Using the band shift assay, we observed the striking result that the binding affinity was affected by the length of the homologous core (Fig. 6a). Whereas RuvC bound ~25% of the input J12, this was reduced to ~2% with J6. Binding to J3, J0 and the Y-junction was below the threshold of detection under these binding conditions. When we assayed for resolution by RuvC (Fig. 6b), we observed that ~21% of J12 was resolved. In comparison, the amount of J6 and J3 resolved was negligible (faint product bands could only be seen on heavily overexposed autoradiograms). We were unable to detect resolution of J0 or the Y-junction. These results indicate that the interaction with RuvC is affected by the presence of homologous DNA sequences.

Discussion

We have described a prokaryotic *in vitro* recombination system and demonstrate that the *ruvC* gene product resolves recombination intermediates made by RecA protein. At the optimal concentrations of RecA and RuvC proteins, most of the available DNA substrates were converted into recombinant products.

FIG. 4 Specificity of RuvC protein for four-stranded DNA recombination intermediates. Reactions containing either 7.5 μ M single-stranded circular ϕ X174 duplex DNA (lanes a-h), or 15 μ M gapped circular ϕ X174 duplex DNA (lanes i-n) and RecA protein (7.6 μ M) were incubated at 37 $^{\circ}$ C for 5 min. To eliminate the need for *E. coli* single-strand binding protein, all reactions were performed in a low- Mg^{2+} buffer (33 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 3 mM ATP, 2 mM dithiothreitol, 5 mM phosphocreatine, 10 units per ml phosphocreatine kinase and 88 μ g ml $^{-1}$ bovine serum albumin). Strand exchange was initiated by addition of $MgCl_2$ to 14 mM followed by 3'- 32 P-labelled *Pst*I-linearized duplex ϕ X174 DNA (8 μ M). After a further 5 min, RuvC was added at the indicated concentrations. Lanes a and i, controls stopped immediately after addition of linear DNA. Lanes b and j, controls stopped after 5 min of strand exchange (that is, at the time of RuvC addition). Lanes c and k, strand exchange reactions lacking RuvC. Lanes d-h and l-n, complete reactions. Incubation of c-h and k-n was for a further 30 min. Reactions were stopped and deproteinized by addition of protease K, SDS and EDTA to 2 mg ml $^{-1}$, 0.5% and 40 mM, respectively, followed by incubation at 37 $^{\circ}$ C for 10 min. 32 P-labelled DNA was visualized by autoradiography following electrophoresis through 0.85% agarose.



Resolution occurred by specific endonucleolytic cleavage and gave rise to products that were characteristic of both recombinant and parental types.

Interestingly, RuvC-catalysed resolution occurred in a reaction in which RecA protein was actively promoting strand exchange. Although we cannot exclude the possibility that resolution occurred in regions that were free of RecA, it is possible that DNA within the nucleoprotein filament is accessible to a small protein such as the 19K RuvC protein. In support of this hypothesis, the resolution bias observed in the presence of RecA protein (Fig. 3), was removed by deproteinization of the intermediates before to addition of RuvC (data not shown).

The RuvC protein shows a high specificity for recombination intermediates and Holliday junction analogues. We have observed cleavage of Holliday junctions made by RecA (Fig. 1), synthetic X-junctions (Fig. 5) and cruciform structures that extrude from the plasmid pCollR215 (unpublished data). In contrast, three-stranded intermediates made by RecA (Fig. 4), synthetic Y-junctions (Fig. 6), single-stranded circular and linear duplex Φ X174 DNA (data not shown), are not digested at

similar protein-DNA ratios. These properties distinguish RuvC from bacteriophage T4 endonuclease VII which cleaves single-stranded DNA²³ and a broad range of structural perturbations in duplex DNA, including cruciforms^{13,14,24}, X junctions²⁵⁻²⁷, Y junctions²⁸, heteroduplex loops²⁹, base mismatches¹⁵, nicks¹⁵ and gaps¹⁵. These differences presumably reflect the specialized role that RuvC plays in the resolution of recombination intermediates compared with T4 endonuclease VII, which debranches DNA before packaging²³. In this regard it might be more closely related to activities that have been partially purified from *Saccharomyces cerevisiae*^{30,31}.

Studies with small synthetic junctions provide some insight into the mechanism of resolution by RuvC. First, resolution does not require RecA protein. The role of RecA protein in the *in vitro* recombination system (Figs 1-3) is to promote homologous pairing and strand exchange, and the recombination intermediates made by RecA serve as substrates for RuvC. Second, resolution is affected by the presence of homologous DNA sequences at the junction point. Using a synthetic junction that contains a central homologous core of 12 bp, we demonstrated specific binding and cleavage by RuvC (Figs 5 and 6). In contrast, similar junctions with homologous cores of 6, 3 or 0 base pairs were not resolved. How does homology affect RuvC-catalysed resolution? It is possible that branch migration is required to bring the crossover point to a DNA sequence that is amenable for cleavage. But this seems unlikely as junction J6 (which is a very poor substrate) contains the DNA sequences that are cleaved in J12. Alternatively, resolution of the junction may require homologous alignment of the two duplexes within the active site of RuvC. Our results do not allow us to distinguish between these and other possibilities.

A role for RuvC in the resolution of recombination intermediates is consistent with the phenotype of *ruvC* mutants^{32,33}. Cells carrying *ruv* mutations are sensitive to DNA-damaging agents (including ultraviolet and γ -irradiation, and various mutagens)³⁴, and are recombination-deficient in *recBCsbcBC*^{32,35}, *recBCsbcA*^{36,37} and *recG*³⁸ genetic backgrounds. But *ruv* single mutants show only slight recombination deficiency during

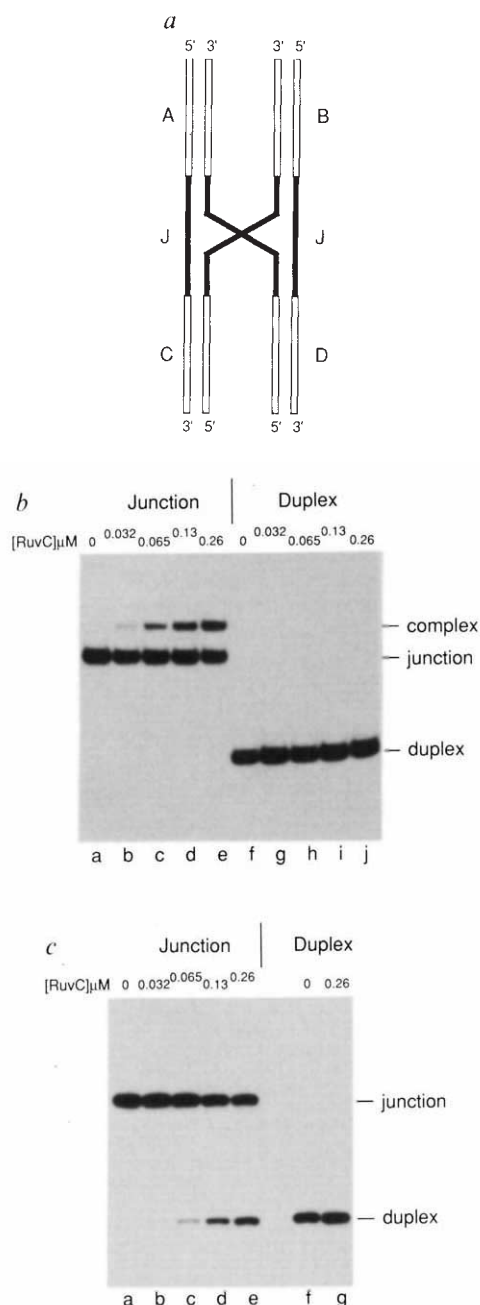


FIG. 5 Binding and resolution of a synthetic DNA junction. *a*, Synthetic junction produced by annealing four single-stranded oligonucleotides. The 12-bp region of homologous DNA sequences (J) is flanked by heterologous sequences (A, B, C and D). *b*, Binding reactions (20 μ l) were carried out on ice for 15 min in 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM dithiothreitol and 100 μ g ml⁻¹ bovine serum albumin. Complexes were assayed by PAGE through a 4% low-ionic-strength gel⁴⁵. *c*, Resolution reactions (20 μ l) were carried out at 37 °C for 60 min in 50 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 1 mM dithiothreitol and 100 μ g ml⁻¹ bovine serum albumin. Samples were deproteinized using SDS and assayed by PAGE through a neutral 10% gel⁴⁵. DNA was detected by autoradiography. Reactions contained $\sim$ 1.8 μ M ³²P-labelled junction J12 (lanes a-e) or linear duplex DNA (lanes f-j). The concentrations of RuvC are indicated. The synthetic junction (J12) and linear duplex DNA have been described¹⁸. The junction was prepared by annealing four oligonucleotides (oligo 1[A-J-C], 5'-³²P-end labelled oligo 2[C-J-B], oligo 3[B-J-D] and oligo 4[D-J-A]). The linear duplex was prepared by annealing ³²P-labelled oligonucleotide 1 with its complementary strand. DNA concentrations, which were only approximate owing to their low concentration, were measured using DNA dipsticks (Invitrogen, San Diego) and are expressed in moles of nucleotide residues. Band intensities on autoradiograms were quantitatively measured using an LKB 2222-10 Ultrascan XL laser densitometer.

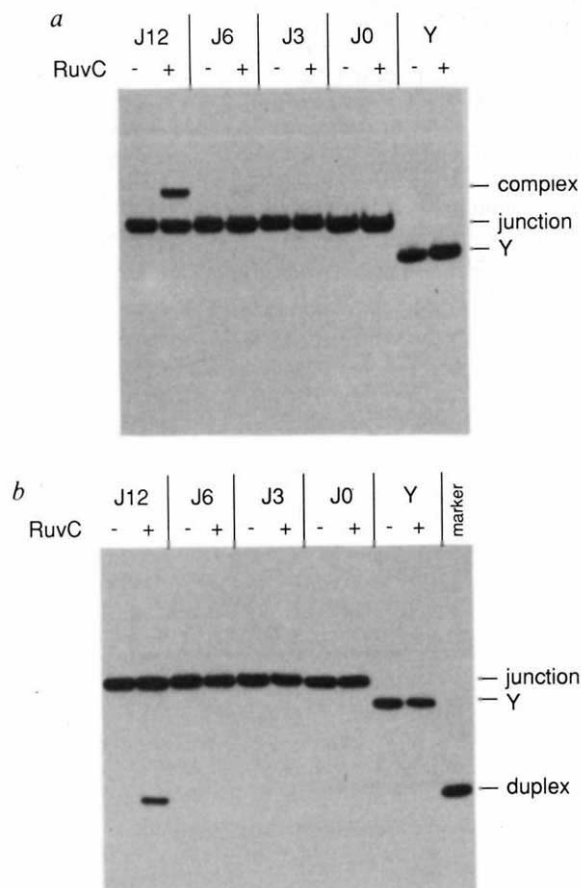


FIG. 6 Resolution of a synthetic junction depends on homology. Junctions (1.8 μ M) were incubated without (–) or with (+) RuvC protein (0.13 μ M) under the appropriate conditions for binding or cleavage as described in the legend to Fig. 5. Binding reactions (a) were assayed by PAGE through a low-ionic-strength gel. Cleavage reactions (b) were assayed by neutral PAGE. 32 P-labelled DNA was detected by autoradiography. The four synthetic junctions (J12, J6, J3, and J0) contain homologous cores of 12, 6, 3 or 0 bp respectively. J12 and J0 have been described^{18,27}. J6 and J3 were designed so that the central core of J12 was reduced to 6 and 3 bp respectively. Oligonucleotide 2 was common to J12, J6 and J3, and was used for 5'- 32 P-labelling before annealing. In addition, the sequences used to prepare J6 were oligonucleotides 1, (5'-GACGCTGCCGAATTCTGGCGTTCTAGGACATCTT-TGCCACGTTGACCC-3'), 3(5'-CAACGTCATAGACGATTACATTGCTAGGAGCTG-CTGTCTAGAGACTATCGA-3'), and 4(5'-ATCGATAGTCTCTAGACAGCAGCTC-CTAGAACGCCAGAAATTCGGCAGCGT-3'). Similarly, J3 required oligonucleotide 1, (5'-GACGCTGCCGAATTCTGGCGTTATAGGACATCTTTGCCACGTTGACCC-3'), 3(5'-CAACGTCATAGACGATTACATTGCTAGACGCTGCTGTCTAGAGACTATCGA-3') and 4(5'-ATCGATAGTCTCTAGACAGCAGCTCTATAACGCCAGAAATTC-GGCAGCGT-3'). The Y-junction contained oligonucleotides 1 and 4 of J12, and the third strand was (5'-TGGGTCAACGTTGGGCAAAGATGTCCGGACATGCTGTCTAGAGACTATCGA-3'). It was 32 P-labelled at the 5'-terminus of oligonucleotide 1.

conjugation or transduction, which may indicate the presence of an alternative resolvase, or alternative pathways for the formation of recombinant DNA.

The *ruv* locus on the *E. coli* chromosome encodes three genes (*ruvA*, *ruvB* and *ruvC*), and mutations in any of these confer *ruv* phenotype³⁹. Nucleotide sequencing shows that *ruvA* and *ruvB* form a single operon regulated by LexA repressor as part of the SOS response to DNA damage^{40,41}. Transpositional insertions in *ruvA* exert a polar effect on *ruvB*. The RuvA (22K) and RuvB (37K) proteins have been purified and shown to interact (the ATPase activity of RuvB protein is stimulated by the presence of RuvA and DNA)^{42,43}. Sequencing of the *ruvC* gene

reveals that it forms an operon with *orf-26* (an upstream gene formerly designated *orf-33*)^{19,39}.

The RuvC protein used here is free of RuvA as it was prepared from a *ruvC* overexpression plasmid grown in a *ruvA60::Tn10* host. The need for relatively high levels of RuvC protein (Fig. 1), coupled with observations that RuvC may be turning over quite slowly, could indicate a requirement for other factors in order to make it catalytic. Possible candidates include RuvA, RuvB or the product of *orf-26*. In conclusion, our results demonstrate a cellular resolution activity that has been linked by genetic observations to a role in recombination and the recombinational repair of DNA damage. □

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Images of atomic carbon in the interstellar medium

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ALTHOUGH carbon is the fourth most cosmically abundant element, it exists only rarely as neutral atoms, because carbon that is cool enough to be neutral rapidly combines with other atoms to form molecules such as CO. Atomic carbon (C I) forms when CO is dissociated by ultraviolet photons, but the dissociation energy is close to the ionization energy of C I, so that neutral carbon is easily ionized into C II. At the edges of molecular clouds, carbon will exist mostly in the form of C II, with only a narrow transition zone containing C I covering the interior of the cloud, where CO predominates. We present here observations of the submillimetre line due to the $^3P_1 \rightarrow ^3P_0$ fine-structure transition of C I, from which we construct high-resolution maps of neutral carbon in molecular clouds in the star-forming region Orion A, in the externally illuminated cloud S140, in the edge-on ionization front of M17, and at the Galactic Centre. C I emission is indeed concentrated in a transition zone, but we confirm earlier suggestions that neutral carbon is also present at lower concentrations within the clouds. In the case of M17, our images provide direct evidence for clumpiness in the cloud distribution.

The observations were obtained at the James Clerk Maxwell Telescope in Hawaii. A new dual-polarization InSb detector (R.P. *et al.*, manuscript in preparation) was used at a frequency of 492.1607 GHz (wavelength $\lambda \approx 609 \mu\text{m}$). The high-altitude site, new receiver and excellent efficiency of the telescope allow observations that have hitherto been impossible. A new mapping technique was used: the local oscillator frequency was held constant, and the telescope raster-scanned in azimuth, whilst simultaneously beam-switching in the scan direction using the nutating sub-reflector. Several such dual-beam maps of each source, with different parallactic angles and chopper throws to give good coverage in two-dimensional Fourier space, were processed jointly using a maximum-entropy algorithm¹ (MEM) to give the final RA-Dec maps. Single-point position-switched spectra were also taken at a number of positions in each source:

these were used to verify that the emission was indeed zero near the edges of the maps, and to check the maps.

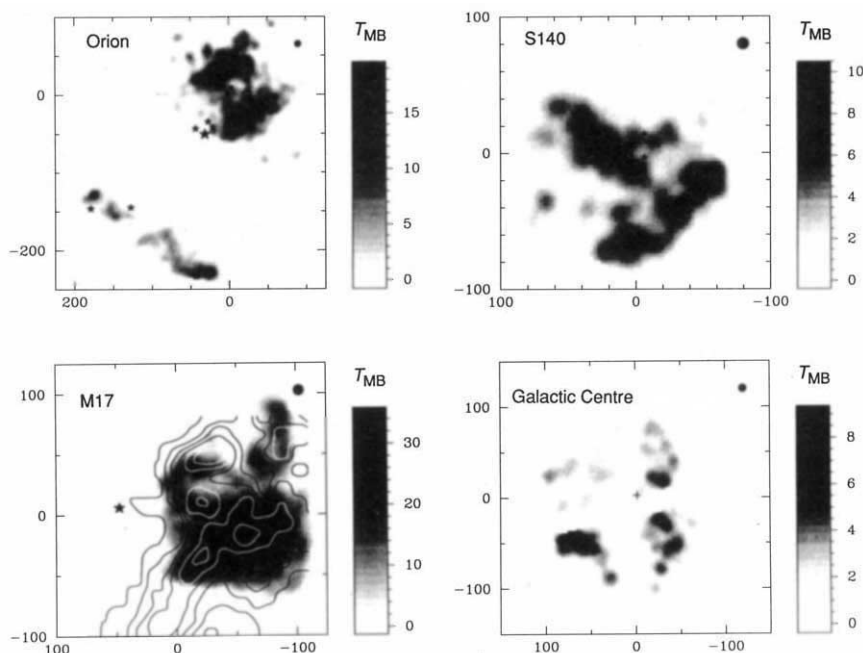
From observations of Mars, we find that the beam size is 9 arcsec, and the main beam efficiency is 0.39 ± 0.04 : this efficiency is consistent with the effective surface error of $\sim 34 \mu\text{m}$ (r.m.s.) deduced from 450- μm beam mapping of Mars, Jupiter and the Moon (R. Hills and J. Richer, personal communication). We deduce from these beam maps that the error beam at 492 GHz should contain $\sim 40\%$ of the total power, and have a maximum amplitude $\sim 2\%$ that of the main beam, with a characteristic radius of ~ 50 arcsec.

The MEM reconstruction process attempts to deconvolve the dual-beam response function from the observed maps. Direct comparison of the dual-beam maps with the position-switched spectra shows that the former underestimate the total flux by up to 30%. This seems to be due to self-chopping of the extended error beam: the error beam was neglected in the MEM restoration because of memory constraints in the computer. The image of the northern part of Orion was reprocessed to take the full error beam into account, using a much more computationally expensive algorithm: we find that the discrepancies with the single-point fluxes are reduced, but that otherwise the image is not qualitatively different from that presented in Fig. 1a.

The Orion molecular cloud contains many indicators of ongoing star formation², including the pre-main-sequence object IRC2, of mass $\sim 50 M_\odot$, which is surrounded by a dense rotating ring of gas. This is being eroded by the combined effects of radiation and an energetic molecular outflow. IRC2 lies ~ 1 arcmin northwest of the well-known Trapezium star cluster, which ionizes surrounding gas to create the prominent optical nebula M42. This cluster also illuminates a dense molecular ridge (the 'Bright Bar'), ~ 3 arcmin southeast of IRC2, where the ultraviolet radiation intensity is $\sim 10^5$ times the average interstellar ultraviolet field, G_0 ($G_0 = 1.7 \times 10^{-3} \text{ erg s}^{-1} \text{ cm}^{-2}$). The C I map obtained towards Orion is shown in Fig. 1a.

In the north of the map, the C I appears as part of a clumpy broken cavity. The two most intense regions lie ~ 30 arcsec (0.06 pc) northeast and southwest of IRC2, at the edges of the 'quiescent ridge', which contains several other star-forming complexes. The C I intensity is weaker along the direction of the molecular outflow (southeast-northwest). Although there is general agreement between the C I distribution and other tracers such as ^{13}CO or C^{18}O , any formal correlation is poor. C I traces the edges of the quiescent molecular ridge instead of following

FIG. 1 C I distribution towards the sources, obtained with a 1.3 km s^{-1} channel (half power width). The positions of infrared and some visible objects are indicated by stars. The centre positions and velocities of the maps are: Orion, RA (1950) 05 h 32 min 46.9 s, Dec (1950) $-05^\circ 24' 26''$ ($+9 \text{ km s}^{-1}$) (the square and dot right of centre are IRC2 and BN respectively, the central four stars are the Trapezium Cluster, and the two at the bottom are Θ^2 Orions A and B); S140, RA (1950) 22 h 17 min 42.0 s, Dec $+63^\circ 3' 45''$ (-8 km s^{-1}) (the stars show the positions of embedded B stars); M17, RA (1950) 18 h 17 min 31.2 s, Dec $-16^\circ 13' 30''$ ($+20 \text{ km s}^{-1}$) (contours of C^{18}O temperature for the same velocity interval as the C I data are overlaid; the star is the position of a bright SAO star); Galactic Centre, RA (1950) 17 h 42 min 29.5 s, Dec $-28^\circ 59' 20''$ ($+50 \text{ km s}^{-1}$) (the Centre is marked with a cross). The beam size is shown as a filled circle in the top right corner. The grey scale is in units of T_{MB} .



the distribution of other molecular species more closely. Southeast of IRC2, the C I forms a narrow bar, containing several clumps embedded in diffuse emission, displaced by ~ 30 arcsec back from the edge of the Orion H II region^{3,4}. The northernmost clumps in the bar lie close to the positions of ionized knots, which resemble Herbig-Haro objects⁵.

Towards IRC2, the main-beam brightness temperature $T_{\text{MB}} = 16.6$ K, and the integrated emission is 79 K km s^{-1} . For an excitation temperature of 100 K, the column density $N(\text{C I}) = 1.1 \times 10^{18} \text{ cm}^{-2}$, and the opacity $\tau(\text{C I}) \approx 0.2$ (see ref. 15 for details of derivation of C I abundance). Assuming $N(\text{CO}) = 1.6 \times 10^{19} \text{ cm}^{-2}$ (ref. 6), $N(\text{C I})/N(\text{CO}) = 0.07$. At the strongest point on the Bright Bar, $N(\text{C I})/N(\text{CO}) = 1.22$. The C I spectrum (Fig. 2a) towards IRC2 is narrow ($\Delta V = 4.5 \text{ km s}^{-1}$), showing no evidence of outflowing molecular gas. Subtracting emission from the ambient cloud, the optically thin upper limit for the outflow gas $N(\text{C I})/N(\text{CO}) < 0.005$ (assuming that the excitation temperature $T_{\text{ex}} = 100$ K). Thus C I seems less abundant in the outflow gas than at nearby cloud positions, as suggested from previous large-beam studies⁷.

S140 is an active region containing a cluster of forming B stars, which appear as compact infrared sources in its core. A nearby (~ 2 pc) B0 star photodissociates the outer layers of the S140 cloud (ultraviolet field $\sim 150 G_0$), exciting a H α emission nebula⁸. The C I map is shown in Fig. 1b. This shows a clumpy bar-like distribution at the front edge of the molecular cloud, adjacent to the ionization front. There is good correlation between this C I bar, running from offsets (+20, -70) to (-60, -10), and a ridge of hot CO (ref. 8). The star-forming core and density peak lie $\sim 50''$ northeast of the C I bar. As shown in Fig. 1b, the infrared sources are adjacent to a clumpy C I ridge deeper inside the cloud. The tendency for C I to avoid the regions of highest column density resembles behaviour seen in Orion. The spectrum towards this core is shown in Fig. 2b. $N(\text{C I})/N(\text{CO}) = 0.19$ at this position, and $N(\text{C I})/N(\text{CO}) = 2.8$ at 30 arcsec west and 45 arcsec south, on the ionization front.

The M17 complex is the archetypal edge-on ionization front, lying next to an O-B star cluster, which heats and provides ultraviolet illumination ($\sim 2 \times 10^5 G_0$) of the cloud edge. In Fig. 1c, contours of the $J=2 \rightarrow 1$ C¹⁸O line for a velocity interval that matches the C I data (J. Stutzki, personal communication) are overlaid on the C I distribution. The C I appears clumpy and fragmented, dropping off sharply (< 1 beamwidth) at the eastern edge, next to the ionization front. The C I peaks show

considerable position offsets from the C¹⁸O peaks; C I emission is not enhanced in the regions of highest column density, or on the ionization front of M17 (see also ref. 9). No significant velocity variation is seen across the edge of the cloud. A spectrum is shown in Fig. 2c.

The inner 10 pc of our Galaxy contain a rotating neutral molecular ring, surrounding a 2-pc-radius ionized cavity. The inner edge of this ring is excited by a strong ultraviolet field ($\sim 7 \times 10^4 G_0$) and hot, dense gas has been reported¹⁰. The C I distribution is shown in Fig. 1d, and a spectrum in Fig. 2d. The C I is concentrated into several clumpy groups lying ~ 2 –4 pc from the Galactic Centre. As in the other sources, the C I peaks lie close to, but do not coincide with, those of other molecular tracers¹¹. The ring has a rotation velocity of $\sim 100 \text{ km s}^{-1}$, so the C I map velocity was chosen to match a dominant velocity seen in the optically thin C¹⁸O line.

The observations reported here represent an advance on earlier observations of C I^{12,13} which showed that $N(\text{C I})/N(\text{CO})$ varied from > 10 in clouds having low extinction (A_v), to ~ 0.1 in cores where $A_v \approx 100$ magnitudes. This contrasted with chemical models^{14,15} where carbon at molecular cloud edges is almost all in the form of C II, with a layer of C I between $A_v \approx 3$ –8, and the transition between C and CO at $A_v \approx 5$ (refs 16, 17), the C II/C I/CO interface existing in a narrow range of hydrogen column densities between 4×10^{21} and 10^{22} cm^{-2} . But C I would rapidly convert back to CO (in $< 10^6$ yr), and multi-component models^{18,19}, in which dense clumps are interspersed with a more tenuous interclump medium, were developed. The C I abundances produced are relatively insensitive to the external ultraviolet field, the C I forming narrow shells around clump cores. In S140, we may be seeing evidence of both processes: direct photodissociation of the cloud edge giving the bright C I rim, and then, deeper into the cloud, penetration of ultraviolet photons through the interclump medium. The present observations show, first, that molecular clouds adjacent to ionization fronts often show strong C I emission from a thin surface layer. In S140 and the Orion bright bar, photodissociation of CO leads to peak values of $N(\text{C I})/N(\text{CO})$ greater than 1, even though this C I layer is only marginally resolved in our 9-arcsec beam. Second, our observations also confirm previous conclusions, based on lower-resolution data, that C I is widely distributed throughout molecular clouds, remaining abundant ($N(\text{C I})/N(\text{CO})$ typically ~ 0.1) up to several parsecs away from the ultraviolet illuminated edges. C I is not detected in the IRC2 outflow, where we find that $N(\text{C I})/N(\text{CO}) < 0.005$, seeming to indicate that shock dissociation of CO in the vicinity of outflows is not a principal source of C I. Finally, they show that in nonuniform molecular clouds such as M17, the C I is a maximum on the edges of the clumps avoided by column density tracers such as C¹⁸O, and apparently avoids the densest regions, presumably because the ultraviolet radiation is too highly attenuated. As the C I emission is distributed throughout the molecular cloud core, this is direct evidence for low-filling-factor, high-density-contrast clumping, as previously deduced from molecular-line and lower-resolution C I observations. $\square$

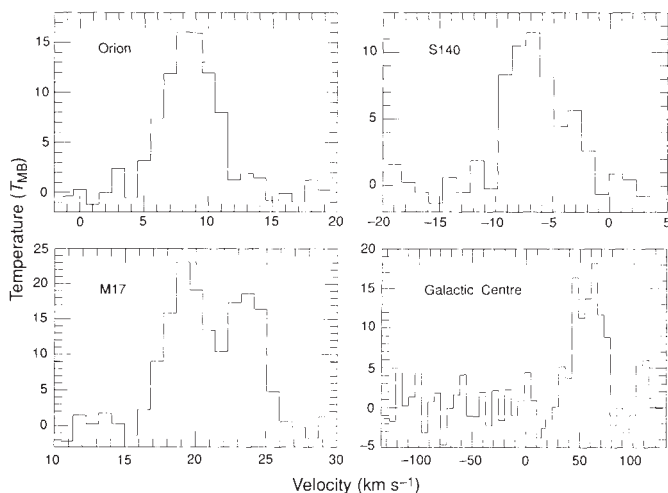


FIG. 2 Position-switched C I spectra towards the sources, at the positions: Orion, RA (1950) 05 h 32 min 47.0 s, Dec (1950) $-05^{\circ}24'23''$; S140, RA (1950) 22 h 17 min, 42.0 s, Dec $63^{\circ}3'45''$; M17, RA (1950) 18 h 17 min 29.9 s, Dec $-16^{\circ}13'00''$; Galactic Centre, RA (1950) 17 h 42 min 27.2 s, Dec $-28^{\circ}59'53''$. The vertical axis is in units of T_{MB} .

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Manufacturable low-noise SQUIDs operating in liquid nitrogen

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THE superconducting quantum interference device (SQUID) holds promise for a wide range of applications, including the measurement of magnetic fields generated by the brain and heart, detection of tiny cracks and corrosion currents, and exploration of oil and mineral deposits. Currently available SQUIDs rely on low-transition-temperature (low- T_c) superconductors, and must therefore be refrigerated to liquid-helium temperature (4.2 K). This difficult cryogenic requirement has presented a barrier to the widespread implementation of SQUID technology. We report here on the development of a high- T_c d.c. SQUID with a noise level in liquid nitrogen (77 K) that is a significant improvement, at frequencies of practical interest, for SQUIDs produced by a manufacturable fabrication process^{1,2}. The energy sensitivity of 1.6×10^{-29} J Hz⁻¹ at 10 Hz is comparable to the best previously reported³ for any high- T_c SQUID at 77 K. These high- T_c SQUIDs are sensitive enough for many of the applications presently possible only with liquid helium devices.

A relatively small number of research groups have fabricated d.c. SQUIDs from thin-film Josephson junctions that operate at liquid-nitrogen temperature¹⁻⁸. The primary difficulty has been in finding a reproducible fabrication process for making SQUIDs that are sensitive enough for practical applications. Previously reported high- T_c SQUIDs suffer from excessive noise at frequencies below 100 Hz, which is the region of interest for the most promising applications including those alluded to above. The lowest noise reported has come from two types of grain-boundary junction, one of which consists of naturally occurring grains in a Ti-Ba-Ca-Cu-O superconducting film³, and the other of which stems from a grain boundary in a Y-Ba-Cu-O film deposited on a bicrystal substrate^{4,5}. The process for fabricating these junctions, however, does not seem suitable for producing more than a small number of SQUIDs. An alternative bi-epitaxial process has been developed to fabricate Y-Ba-Cu-O grain-boundary junctions in larger numbers, although the reported noise performance for the SQUIDs using

these junctions^{1,2} is not as good as that for the most sensitive grain-boundary junctions.

The SQUIDs we report here use a different type of Josephson junction, namely a superconductor/normal metal/superconductor (SNS) junction, to achieve substantially improved noise performance from a practical fabrication process. The process used to make these junctions is highly reproducible⁸. Apart from experimental devices made to test the limits of the manufacturing process, over 100 devices (>95% yield) have been fabricated that demonstrate the expected Josephson-junction behaviour.

The junction structure is shown in Fig. 1. The fabrication begins by etching a sharp step in a LaAlO₃ substrate. The high- T_c superconductor, YBa₂Cu₃O_{7- δ} , is deposited across the step using off-axis single-target sputtering. The sharp step-edge produces a break in the superconducting film and exposes the two edges of the film. The normal metal is sputter-deposited *in situ* (to promote a clean interface) such that it covers the step and joins the two superconducting films. We have successfully used silver as well as a silver-gold alloy for the normal metal. The junction width, typically 5 μ m, is defined using standard photolithography followed by ion-beam etching, making the process compatible with routine production methods. More fabrication details can be found elsewhere⁸.

The reproducibility of the process stems directly from using a step to generate a precisely defined small gap in the

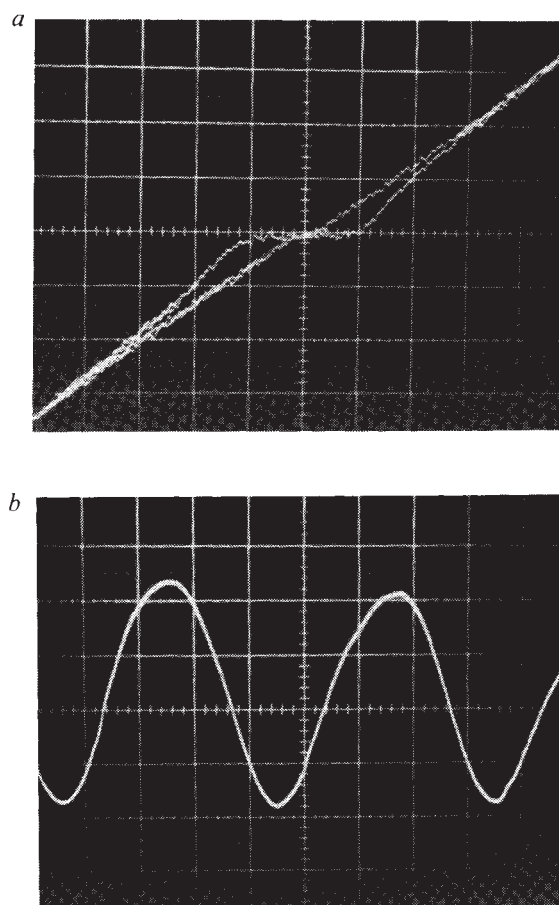


FIG. 2 *a*, Current-voltage characteristic of a d.c. SQUID measured at 77 K with and without an applied magnetic field. For an applied magnetic flux of $\Phi_0/2$, the critical current is essentially entirely suppressed, whereas for zero applied field, $I_c = 22$ μ A. Vertical scale: 10 μ V per division. Horizontal scale: 20 μ A per division. *b*, SQUID output voltage as a function of applied magnetic field. The SQUID is biased at a current just above the zero-field critical current. Vertical scale: 2 μ V per division. Horizontal scale: 30 mG per division.

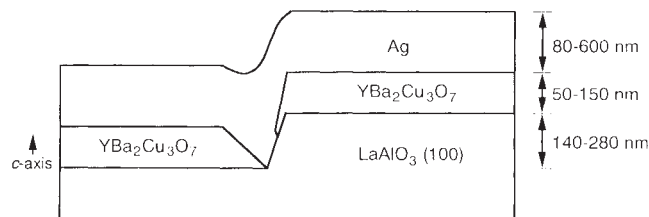


FIG. 1 Schematic diagram of the Josephson junction structure. The superconducting film is epitaxially grown on the substrate such that the *c*-axis of the crystal lattice is perpendicular to the plane of the substrate. Note the material exposed at the film edge near the step consists of *a*-axis oriented superconductor, which has a longer superconducting coherence length and hence a more favourable conduction path.

TABLE 1 Measurements at 77 K for three SQUIDs

	L (pH)	$\partial V/\partial \Phi$ ($\mu\text{V}/\Phi_0$)	$S_{\Phi}^{1/2}(\mu\Phi_0\text{Hz}^{-1/2})$			ϵ meas (J Hz^{-1})			ϵ_w theor (J Hz^{-1})
			10 Hz	40 Hz	100 Hz	10 Hz	40 Hz	100 Hz	
SQUID A	30	17	34	17	11	8.3×10^{-29}	2.1×10^{-29}	8.6×10^{-30}	4×10^{-31}
SQUID B	15	6	13	6.5	*	2.4×10^{-29}	6.0×10^{-30}	*	7×10^{-30}
SQUID C	15	17.5	10.5	4	*	1.6×10^{-29}	2.3×10^{-30}	*	7×10^{-31}

The SQUIDs are those in Fig. 3. Asterisks indicate that the SQUID noise was below the system noise floor. The calculated white-noise energy sensitivity from equation (3) is also given.

superconductor. The gap spacing is fixed by the height of the step, along with the superconductor film thickness, and determines the strength of the Josephson coupling through the proximity effect in the normal metal⁹.

Current-voltage characteristics revealing a well-defined critical current, I_c , have been measured from 4.2 K to over 77 K on nearly all devices. The highest temperature at which a critical current has been observed on these devices is 87 K. Normal-state resistances for the devices, R_n , can be controlled over a wide range, from 0.002 to 2Ω . A key figure of merit, the $I_c R_n$ product, is typically 0.2–2 mV at 4.2 K, although values up to 4.6 mV have been achieved.

Representative electrical characteristics at 77 K of a d.c. SQUID made from these junctions are shown in Fig. 2. The modulation of the critical current with magnetic field reveals nearly ideal behaviour. The SQUID inductance, L , is calculated to be 15 pH, leading to a self-shielding parameter, $\beta = 2LI_0/\Phi_0 \approx 0.16$, where $I_0 = I_c/2$ is the critical current of the individual junctions comprising the d.c. SQUID, and Φ_0 is the magnetic flux quantum. The highest $I_c R_n$ we have observed at 77 K is 0.6 mV, although typical values range from 10 to 150 μV .

We have measured the magnetic flux noise by biasing the SQUID near its critical current and amplifying the voltage output with a low-noise transformer followed by a low-noise preamplifier. An external wire-wound magnet coil was used to

bias the SQUID in open-loop fashion on an appropriate point of the curve of voltage against applied magnetic flux. The voltage noise, $S_V^{1/2}$, was typically measured from 1 to 500 Hz with a spectrum analyser. The transfer function, $\partial V/\partial \Phi$, was measured to obtain the flux noise¹⁰:

$$S_{\Phi}^{1/2} = \frac{S_V^{1/2}}{\partial V/\partial \Phi} \quad (1)$$

The energy sensitivity, ϵ , of the SQUID is obtained from the spectral density of the flux noise, S_{Φ} , and is given by:

$$\epsilon = S_{\Phi}/2L \quad (2)$$

The gain of the transformer–preamplifier stages was calibrated as a function of frequency, and the system noise, including transformer, preamplifier, measurement probe and cables, was also precisely measured. The system noise, typically $\sim 100 \text{ pV Hz}^{-1/2}$ from 5 to 100 Hz, was subtracted from the measurement data in the conventional fashion to determine the contribution of the SQUID alone. The system noise contribution was small at lower frequencies such as 10 Hz; for the lowest-noise SQUIDs, the system noise dominated at frequencies above 40 Hz. Although flux-locked loop operation has not yet been attempted, the stable SQUID output characteristics such as shown in Fig. 2b indicate that this should not be difficult.

The energy sensitivity at 77 K for three different d.c. SQUIDs is shown in Fig. 3. SQUID A is representative of seven different devices that we have measured, whereas B and C are the lowest-noise devices that we have measured so far. SQUID parameters and measurement data are summarized in Table 1. The inductances are calculated from the geometry of the d.c. SQUID and are reasonably consistent with the inductance determined from the depth of the modulation¹¹. At low frequencies, the energy sensitivity of devices B and C is 50 times lower than that reported previously for SQUIDs produced by a manufacturable fabrication process¹. The data indicate that the noise in all three of the SQUIDs is dominated by $1/f$ noise below 100 Hz. The noise varies as $1/f^\alpha$ where $\alpha = 1.0, 1.1$ and 1.3 for SQUIDs A, B and C respectively. The source of the $1/f$ noise is unknown at present; it may stem from fluctuations in the critical current of the junctions or from flux noise generated in the superconducting material connecting the two junctions.

Although measurement system noise prevented us from obtaining white-noise data, it is still of interest to compare our highest-frequency measurements with the calculated white noise. The white noise is important in that it represents the best noise performance that can be expected from a SQUID with a given set of design parameters. The theoretical value of the white-noise energy sensitivity for values of $\beta \ll 1$ is given by¹¹:

$$\epsilon_w \approx \frac{k_B T L}{\beta^2 R} \quad (3)$$

where k_B is Boltzmann's constant, T is temperature and R is the resistance of each junction in the d.c. SQUID. For SQUID C, we calculate the white noise $\epsilon_w = 7 \times 10^{-31} \text{ J Hz}^{-1}$, which is only about one-third of the value $\epsilon = 2.3 \times 10^{-30} \text{ J Hz}^{-1}$ measured at 40 Hz. As it is reasonable that the $1/f$ dependence holds to higher frequencies, we expect that above 40 Hz the noise should approach the calculated white noise value even more

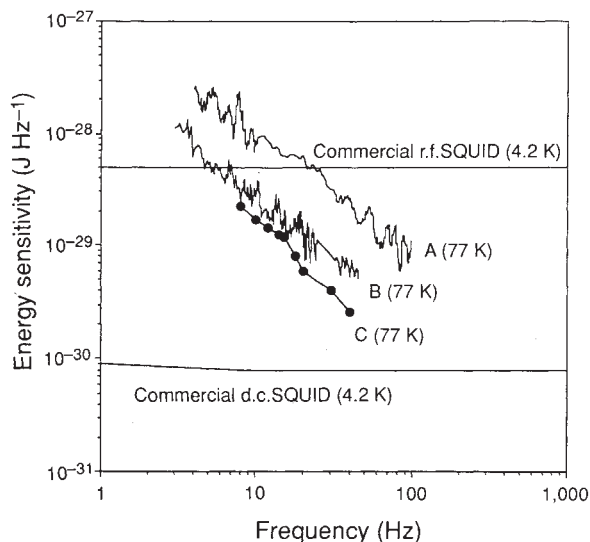


FIG. 3 Energy sensitivity as a function of frequency for three high- T_c d.c. SQUIDs (A, B and C) at 77 K. The normal metal for all SQUIDs is a silver–gold alloy. The measurement system noise level limited the data collection to the frequencies plotted, and SQUID C was measured at the fixed frequencies shown. The energy sensitivities of niobium-based commercial SQUIDs (Bio-magnetic Technologies) operating at 4.2 K are shown for comparison. The low- T_c SQUID noise characteristics are dominated by white noise across most of the frequency range and hence appear different from those of the high- T_c SQUIDs that are dominated by $1/f$ noise. The low $1/f$ noise in the commercial d.c. SQUID stems in part from the use of an electronic biasing scheme designed to reduce $1/f$ noise. Note that the noise performance of the commercial r.f. SQUID at 4.2 K is sufficient for many applications.

closely. The measured value of the transfer function $\partial V/\partial \Phi = 17.5 \mu\text{V}/\Phi_0$ for SQUID C is also close to the low- β theoretical estimate¹¹ given by $\partial V/\partial \Phi \approx \beta R/L = 20 \mu\text{V}/\Phi_0$.

Note that none of our devices is optimized for the best noise performance, which is expected¹¹ for values of $\beta \approx 1$. In this regime¹², $\varepsilon_w \approx 9k_B T_L/R$, indicating that the white noise of SQUID C might be improved on by a factor of 5 if a SQUID with a higher critical current at 77 K was used. We have fabricated junctions with higher critical currents by using a shorter step, although the present process is not optimized to produce such devices routinely. Additional improvement to achieve the ultimate noise sensitivity could also result from further increasing device resistance. Individual junctions with resistances as high as 5Ω have been produced.

Finally, for the SQUID to be used as a sensitive detector of magnetic field, it will need to be coupled to a high- T_c flux transformer, a number of which have recently been reported^{1,2,13}. Efficient coupling favours an increase in SQUID inductance by a factor of 3 to 5 above our current inductances, which should be easy to obtain by increasing the loop dimensions. The impact of increasing the SQUID inductance on $1/f$ noise is not,

however, straightforward, because of the limited understanding of $1/f$ noise in SQUIDS¹⁴. For example, if the $1/f$ noise arises from thermally activated motion of magnetic flux vortices in the SQUID loop (flux noise), then an increase in the SQUID inductance will lead to a higher noise level. On the other hand, there is evidence to indicate that at temperatures more than a few degrees below T_c , the $1/f$ noise in high- T_c SQUIDS made from high-quality (that is, high-critical-current density) films may stem from fluctuations in the critical current of the junctions^{4,15,16}. The energy sensitivity then scales inversely with SQUID inductance, provided that the $1/f$ noise can be modelled in a similar fashion as for low- T_c SQUIDS¹⁷. This also assumes that the critical current is adjustable independently from the inductance in order to optimize SQUID operation. Importantly, our step-edge junction fabrication process allows the critical current to be independently set over a wide range, as the gap spacing that determines the strength of the Josephson coupling is readily controlled. In any case, if the $1/f$ noise results from fluctuations in the critical current, then the use of an electronic biasing scheme should reduce this noise, as is achieved for low- T_c SQUIDS¹⁷. □

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A silicon neuron

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By combining neurophysiological principles with silicon engineering, we have produced an analog integrated circuit with the functional characteristics of real nerve cells. Because the physics underlying the conductivity of silicon devices and biological membranes is similar, the 'silicon neuron' is able to emulate efficiently the ion currents that cause nerve impulses and control the dynamics of their discharge. It operates in real-time and consumes little power, and many 'neurons' can be fabricated on a single silicon chip. The silicon neuron represents a step towards constructing artificial nervous systems that use more realistic principles of neural computation than do existing electronic neural networks.

The electrical properties of nerve cells enable brain circuits to perform the prodigious feats of computation that make intelligible the torrent of sensory information confronting us each second. The electrical behaviour of each neuron is determined by combinations of voltage-, ion- and neurotransmitter-sensitive conductances, which control currents of various ions across the membrane. These ion currents determine the voltage of the cell membrane and hence the electrical properties of the nerve cell. Here we use combinations of complementary metal-oxide-semiconductor (CMOS) circuits, fabricated in very-large-scale integrated (VLSI) technology, to represent the different ion

currents. Together they form the silicon analogue of a biological neuron. This realistic 'neuron' is a considerable departure from neural-net hardware^{1,2}, which implements mathematical or engineering abstractions of neurons.

Neurons communicate with each other using nerve impulses, which are self-regenerating spikes of the membrane voltage. During a nerve impulse, several different conductances are activated in the membrane, which allow selected ions to flow down the voltage gradient between the membrane voltage and the equilibrium potential of the ions concerned; the equilibrium potential of each ion is determined by its relative concentration on either side of the semipermeable cell membrane. Our circuits emulate ion currents in neurons of the neocortex³, which is the principal region of high-level processing in the brain. The nerve impulse itself is generated by a current carried by sodium ions (INA, see Fig. 1 legend for explanation of abbreviations) and a current carried by potassium ions (IKD, delayed rectifier current). The rate at which impulses are produced is controlled by two further potassium currents with slower dynamics, the so-called A-current (IKA) and the calcium-dependent potassium current (IAHP, after-hyperpolarizing current). The activation and inactivation of the membrane conductances that control these currents are both time- and voltage-dependent. The voltage-dependent conductances of biological membranes in steady state have a sigmoidal conductance-voltage relation⁴ which is similar to the current-voltage relation generated by CMOS transistors arranged to form a 'differential pair'⁵. We have exploited this analogy between silicon devices and biological membranes.

In the circuits of the silicon neuron, the cell membrane is represented by a fixed capacitor and variable leak conductance. The conductances in the membrane and their control mechanisms are represented by individual circuits of the kind shown in Fig. 1. The basic principle of these circuits is that the output

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current of a differential pair is used to control a 'conductance' transistor, which represents the membrane conductance of the ion concerned. Thus, in this analogy, the activation (or inactivation) of the membrane conductances is represented by the time- and voltage-dependent output currents of differential pairs. The maximum value of each 'activation' current is set by the bias transistor associated with the differential pair. The knee transistor of the pair determines the 'membrane' voltage at which the conductance is activated. As the 'membrane' voltage applied to the activation transistor of the pair increases with respect to the knee voltage, so the activation current increases sigmoidally towards its maximum value. The activation current controls the 'conductance' transistor. This transistor regulates the flow of 'ion' current down the gradient between the 'membrane' voltage and the 'equilibrium potential' of the ion. The conductance transistors are fabricated with dimensions that make their behaviour more ohmic than standard CMOS transistors. The ohmic behaviour is not ideal, however, and this causes the small deviation between real and analogue performance noted in Fig. 1c. The time dependence of the analogue conductances is achieved by using follower-integrators with variable time constants to act as low-pass filters for the membrane voltage (Fig.

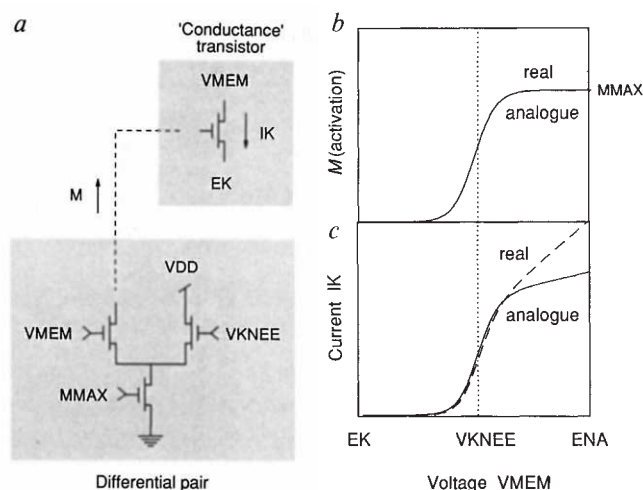


FIG. 1 A CMOS circuit and the biological voltage-dependent ion current it represents. Acronyms are given in capitals to emphasize that they are analogue signal names and to avoid confusion with equivalent names in the biological literature, which are usually given as subscripts; where necessary, analogue concepts appear in quotes to avoid ambiguity. *a*, Basic circuit used in the silicon neuron to emulate ion currents. The circuit consists of an 'activation' and a 'knee' transistor which form a differential pair, their associated bias transistor, and a 'conductance' transistor. The gate voltages of the activation and knee transistors are controlled by the 'membrane' voltage VMEM and a reference voltage VKNEE respectively. The voltage MMAX, applied to the bias transistor, sets the bias current, which is the sum of the currents through the two transistors of the differential pair. When VMEM is very small with respect to VKNEE, the bias current is supplied entirely from the power supply VDD and so the output current M is zero. But as VMEM increases with respect to VKNEE, current M increases sigmoidally towards the maximum bias current set by MMAX (ref. 5). When VMEM = VKNEE, M is half its maximum value. *b*, The form of this current-voltage relation of the differential pair is analogous to the conductance-voltage relation of a real ionic conductance. Thus, M can be used as a conductance 'activation' signal to control (through intermediate circuitry indicated by broken line in *a*) the 'conductance' transistor, which represents the membrane ion conductance. For example, the curve shown here is typical of a potassium conductance that is activated as 'membrane' voltage VMEM increases from EK ('potassium equilibrium potential') toward ENA ('sodium equilibrium potential'). *c*, The potassium current (broken line) that flows through the membrane of biological neurons is the product of conductance M and driving potential (VMEM - EK). Similarly, the analogue 'potassium' current (IK in *a*, solid line in *c*) flows down the gradient between VMEM and EK through the conductance transistor controlled by M. The real and analogue currents agree well except at large driving potentials, where the analogue current has a shallower slope.

2a). The filtered outputs then control the activation (or inactivation) transistors. Some ion currents, such as IKD, are not inactivated and so require only an activation signal (Fig. 2a). Others, such as INA, require both activation and inactivation subcircuits (Fig. 2b). In these cases the activation and inactivation signals combine to control the gate voltage of the conductance transistor.

The silicon neuron comprises a somatic compartment and a dendritic load whose passive electrical characteristics conform to those of a neocortical pyramidal neuron. Active conductances are located only in the somatic compartment. The performance

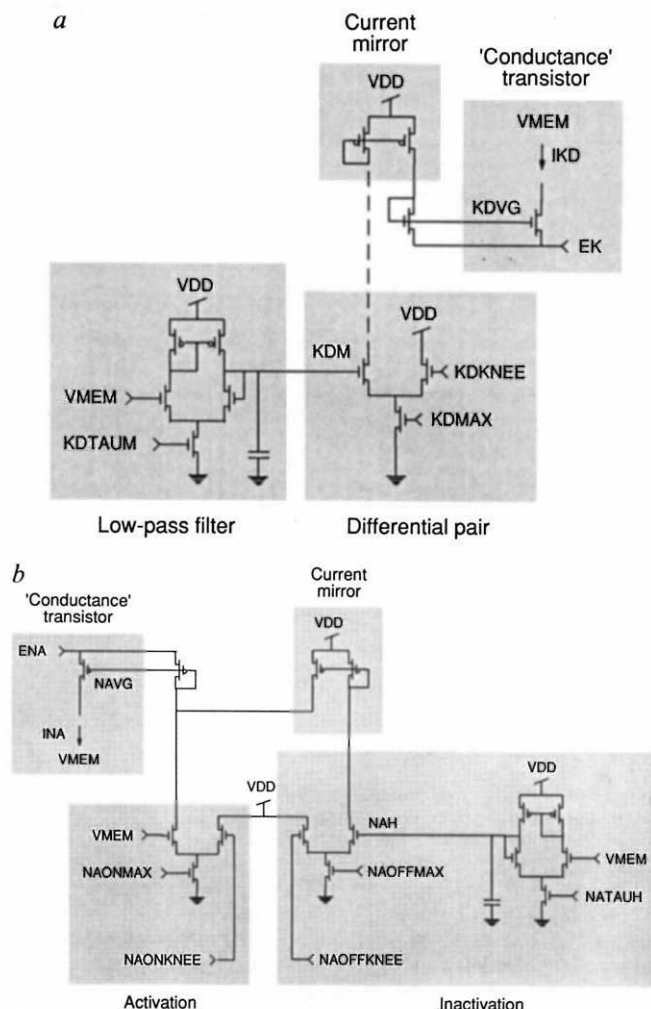
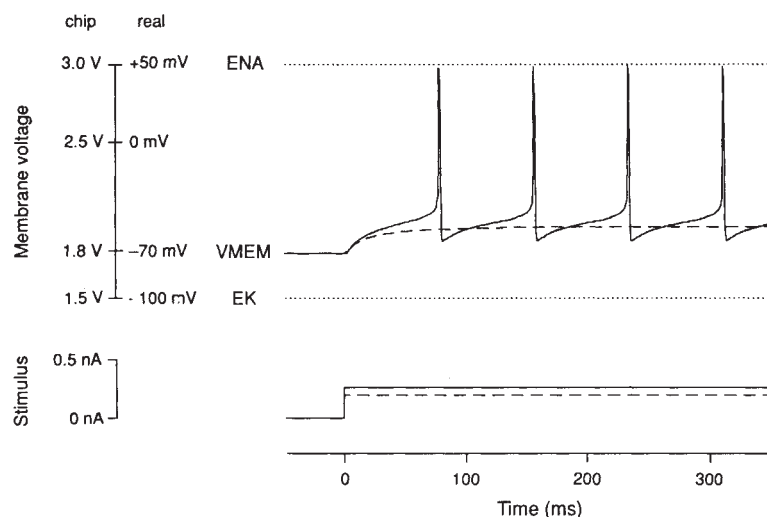


FIG. 2 CMOS circuits that generate the ion currents of the action potential. *a*, Circuit generating the 'potassium' current IKD. 'Membrane' voltage VMEM is low-pass filtered by a follower-integrator whose output KDM controls the activation transistor of a differential pair. The current through this transistor represents potassium 'activation' (broken line). It is reflected through a current mirror (a technical requirement) and transformed into gate voltage KDVG that controls the 'conductance' transistor. This transistor drains the analogue 'potassium' current IKD from the 'membrane' voltage VMEM to the 'potassium equilibrium potential' EK. KDKNEE, half-activation membrane voltage; KDMAX, maximum activation; KDTAUM, activation time constant; VDD, supply voltage. *b*, Circuit generating the sodium current INA. Unlike IKD which does not inactivate, INA depends on the interaction of activation and inactivation subcircuits. INA activates rapidly and so VMEM is applied directly to the differential pair of the activation subcircuit. The output current from this subcircuit represents sodium activation. In the inactivation subcircuit VMEM is low-pass filtered to produce NAH, which controls inactivation differential pair. Activation and inactivation signals combine to yield conductance control voltage NAVG. The 'conductance' transistor drains the analogue 'sodium' current INA from the 'sodium equilibrium potential' ENA to the 'membrane' voltage VMEM. NAOFFKNEE, half-inactivation voltage; NAOFFMAX, maximum inactivation; NAONKNEE, half-activation membrane voltage; NAONMAX, maximum activation; NATAUH, inactivation time constant.

FIG. 3 Response of silicon neuron to 'intracellular' current injection. A subthreshold current stimulus (0.20 nA, broken line) caused a simple charging response of the 'membrane'. A suprathreshold stimulus (0.26 nA, solid line) evoked a discharge of nerve impulses whose form was the same as that measured in actual neurons. Only sodium and potassium impulse currents (INA, IKD) were present in this example, and therefore no adaptation occurred. The 'membrane' voltage VMEM can range between the limits set by the potassium (EK) and sodium (ENA) 'equilibrium potentials'. In real neurons these limits are ~ -100 mV and $\sim +50$ mV respectively (right of ordinate). Biological conductances are ~ 10 times as sensitive to voltage as CMOS transistors^{4,5}, however, and the silicon neuron is driven by a 0–5-V power supply. So for convenience we set the analogue EK = 1.5 V and ENA = 3.0 V (left of ordinate). This choice provides 10 times as large a range of 'membrane' voltage and is roughly centred with respect to the power supply.



of this silicon neuron was evaluated by examining the response of its 'membrane' voltage to 'intracellular' current steps. Instrumentation circuits fabricated on the chip were used to apply external test signals and monitor test points. We used test methods similar to those used in recording from actual neurons, to facilitate comparison of silicon with biological data. When

current steps were injected into the silicon neuron, its response was essentially the same as that of real neocortical neurons (Fig. 3). Subthreshold current stimuli evoked a simple passive charging response, whereas suprathreshold stimuli produced a discharge of nerve impulses. One minor difference is that the peak of the impulse of the silicon neuron is closer to the equilibrium potential for sodium (ENA) than in real neurons because of the deviation from ideal behaviour noted in Fig. 1. This deviation is quantitative rather than qualitative, and its practical effects are minimal.

We used analogues of two potassium currents, IKA, and IAHP to control the rate of impulse production. In actual neurons, IKA slows the re-depolarization that follows each impulse, and this allows the neuron to produce impulses more slowly than would be the case if the membrane recharged passively. The conductance controlling IKA is initially activated by depolarization. This conductance slowly inactivates and allows the membrane to relax gradually toward the spike threshold. The conductance is rapidly de-inactivated following an impulse, and the cycle repeats. The circuit that generates IKA is similar to that of INA (Fig. 2b) except that 'inactivation' is reset by the occurrence of each impulse.

The circuits described above represent voltage-dependent currents. The calcium-dependent potassium current (IAHP) presents the additional problem of representing intracellular calcium concentration. During each nerve impulse, calcium enters the cell through voltage-sensitive calcium channels. If the impulse rate is high, then not all the calcium entering the cell can be buffered sufficiently quickly. The intracellular calcium concentration rises and activates IAHP, which opposes the excitatory input current. This causes the impulse frequency to decrease, or adapt, in the face of a sustained input.

The IAHP circuit represents the 'calcium concentration' as the output voltage across the capacitor of a leaky integrator and this voltage is the activation signal that controls the analogue potassium ion current. Each nerve impulse contributes a small amount of charge, representing calcium entry, to the integrator. Between impulses the output voltage decays toward a resting value. If the impulse frequency is high, the output of the integrator increases and activates IAHP to produce adaptation.

The adaptation of the impulse frequency during sustained inputs and the current/impulse-frequency relationship of the silicon neuron were similar to those observed in neocortical neurons (Fig. 4). Adjustment of the IKD, IKA and IAHP parameters resulted in discharge patterns that were commensurate with the discharge types³, and their variations, observed in real neurons. Like real cells, the discharge inactivates at very large input currents. These robust qualities suggest that the

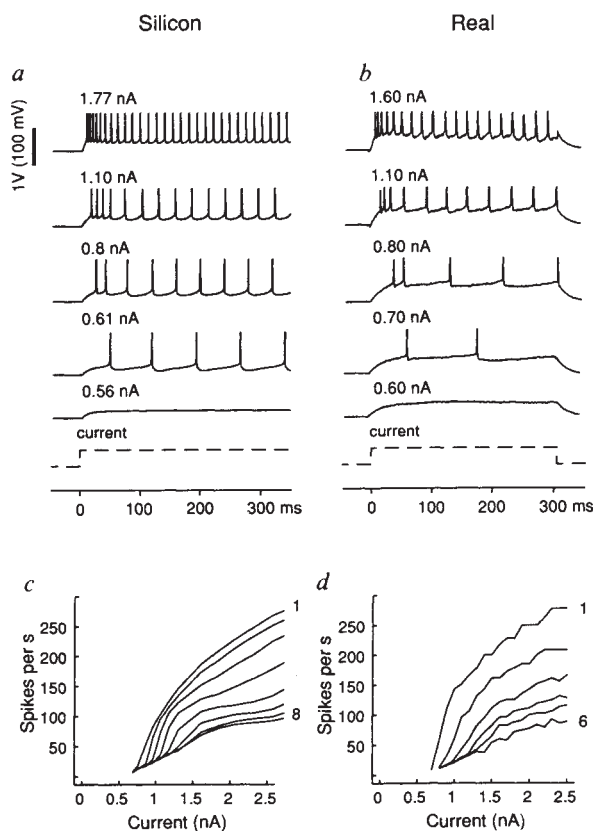


FIG. 4 Comparison of response to intracellular current injection of the silicon neuron and a neocortical neuron. Voltage scale represents 1 V for the silicon neuron and 100 mV for the neocortical neuron. *a*, For small suprathreshold current inputs (0.61 nA), the interimpulse intervals of silicon neuron are dominated by IKA: discharge frequency is low and little adaptation occurs. Larger inputs activate IAHP, which causes adaptation over the first few intervals. *b*, Adaptation in neocortical neuron recorded *in vitro* in cat visual cortex. Nerve impulse amplitudes truncated in *a* and *b*. *c*, Current-discharge relation of first eight interspike intervals of the silicon neuron. The relation of first interval has initial steep slope, then saturates. The fully adapted relation is nearly linear, with a shallow slope. *d*, Current-discharge relation for the first six intervals of the real neuron shown in *b*.

silicon neuron will also function appropriately when incorporated in large networks.

We have shown here that realistic neuronal behaviour such as action potentials and adaptation can be readily incorporated in hardware that operates in real time and that the resulting performance is robust. Unlike existing neural network hardware, the basic circuits of the silicon neuron directly emulate biological behaviour, rather than interpreting the biology in terms of conventional digital or linear design principles. The cost of this realistic behaviour is low because the silicon neuron emulates the characteristics of the biological system directly in its device physics^{5,7}. This allows efficiency in circuit construction. For example, the circuits that generate the action potential together contain fewer elements than a conventional operational amplifier. The power dissipation of the whole circuit including instrumentation amplifiers is only 60 μ W, which compares favourably with the 500-mW dissipation of a typical operational amplifier.

The structural and functional details of single silicon neurons can be increased using building blocks similar to those described above. The same design principles can be applied to any new ionic currents that might be necessary for expressing additional properties of neocortical neurons, or for making 'neurons' of other brain areas. The analogue circuits are compact. The entire silicon neuron, which can be further optimized, occupies less than 0.1 mm². We estimate that a linear array of 100–200 such neurons could be fabricated on a 1 cm $\times$ 1 cm die. The second dimension of the array would contain the dendritic structure and synapses of each neuron.

In principle, the silicon neuron could operate $\sim 10^6$ times as fast as its biological counterpart. We think it important, however, that the dynamics of the biological and silicon neurons be well matched, because the silicon neurons will be used to build machines that interact with real-world events in the same way as biological nervous systems. $\square$

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Complementary Ti and Zr anomalies in orthopyroxene and clinopyroxene from mantle peridotites

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FROM the observation that clinopyroxenes in lherzolites from sub-arc, sub-continental and sub-oceanic lithosphere are depleted in titanium and zirconium, Salters and Shimizu¹ inferred the existence of a worldwide layer in the upper mantle depleted in these 'high-field-strength' elements. This seems inconsistent, however, with the fact that basalts generated in the upper mantle, except in certain restricted environments (related to subduction processes), do not show depletion of these elements. In an attempt to address this conflict, we have used ion-probe measurements to study Ti and Zr abundances in sub-continental spinel peridotites from Liguria, Italy. We confirm the existence of Ti and Zr depletion in clinopyroxenes, but find that these are compensated by enrichment in the coexisting orthopyroxene. The calculated whole-rock abundance patterns therefore show negligible anomalies. We conclude that Ti, Zr and rare-earth abundances in clinopyroxene alone cannot be used to infer the existence of a global mantle layer depleted in high-field-strength elements, a feature that would place important constraints on models of lithospheric growth².

Depletion of high-field-strength elements (HFSE) is widespread in mantle clinopyroxenes^{1,3}. The layer of HFSE-depleted mantle inferred from this depletion has been considered to be a geochemical feature with important petrologic implications. Salters and Hart⁴ have re-emphasized the ubiquity of an HFSE-depleted component in the shallow mantle, as inferred from an extensive compilation of clinopyroxene data (their Fig. 9). Salters and Shimizu¹ defined Ti and Zr depletions by their

normalized abundances relative to the 'neighbouring' rare earth elements (REEs), namely Nd and Sm for Zr, and Eu and Tb for Ti. They determined the order of compatibility of these elements on the basis of both the smoothness of the normalized trace element patterns of MORBs (mid-ocean-ridge basalts)⁵, and the distribution coefficients in the diopside–melt system^{6–11}.

The existence of such depletions raises two important questions. The first concerns the mechanism by which clinopyroxene becomes depleted in Ti and Zr. Assuming that Ti, Zr and REEs have similar diopside–melt partition coefficients, negative Ti and Zr anomalies cannot be produced by simple igneous processes controlled by the diopside–liquid partitioning. Salters and Shimizu¹ therefore argued that high-pressure mineral phases such as perovskite or majorite and garnet^{12,13}, could fractionate HFSE from REE during solid–melt partitioning. The second and more serious problem is that negative HFSE anomalies are inferred (from the clinopyroxenes) for the bulk abyssal peridotites, but are not found in the associated MORBs. The processes relating MORBs and abyssal peridotites are still unclear, but must be more complex than simple equilibrium melting³.

Salters and Shimizu¹ made the critical assumption that the REE and HFSE abundance patterns of clinopyroxenes from spinel lherzolite adequately represented the patterns in bulk rocks, an assumption which is probably correct for REEs^{14–16} but is less well documented for HFSEs. Accordingly, they

TABLE 1 Major element composition of selected Northern Apennine peridotites

	ER-R3/3	ER-N1/4
SiO ₂	43.19	42.32
TiO ₂	0.11	0.12
Al ₂ O ₃	3.21	3.47
Fe ₂ O ₃	8.49	8.80
MnO	0.12	0.11
MgO	38.37	37.37
CaO	2.54	2.83
Na ₂ O	0.17	0.27
H ₂ O ⁺	4.30	3.92
Total	100.50	99.21
Zr (p.p.m.)	10	9

Bulk rocks analysed by X-ray fluorescence.

TABLE 2 Trace element concentrations of clinopyroxene, orthopyroxene and bulk rock in Northern Apennine peridotites

Mode	ER-R3/3				ER-N1/4			
	Cpx (11%), Opx (30%), Ol (58%), Sp (1%)				Cpx (12%), Opx (31%), Ol (55%), Sp (2%)			
	Cpx	Opx	BR1	BR2	Cpx	Opx	BR1	BR2
Ce	5.5	0.020	0.78	0.56	7.7	0.086	1.10	0.79
Nd	5.2	0.049	0.74	0.53	7.3	0.15	1.07	0.78
Zr	45	2.5	6.9	5.2	45	6.6	8.1	6.4
Sm	1.87	0.025	0.27	0.19	2.6	0.068	0.38	0.28
Eu	0.86	0.014	0.12	0.09	1.03	0.025	0.15	0.11
Ti	3,440	725	684	562	3,500	1,090	793	675
Dy	3.2	0.09	0.47	0.34	3.6	0.26	0.58	0.44
Er	2.0	0.10	0.30	0.23	2.2	0.26	0.38	0.30
Yb	1.83	0.12	0.29	0.22	2.0	0.33	0.37	0.30
Zr/Zr*	1.01	4.9	1.09	1.13	0.72	4.5	0.89	0.97
Ti/Ti*	0.57	4.9	0.77	0.87	0.50	3.0	0.73	0.83

All concentrations are in p.p.m. Bulk-rock abundances have been calculated assuming, for BR1, $\text{opx/cpx} = 2$ ($\text{opx} = 28\%$; $\text{cpx} = 14\%$), and for BR2, $\text{opx/cpx} = 3$ ($\text{opx} = 30\%$; $\text{cpx} = 10\%$); in both cases $\text{Zr}^* = (\text{Sm}_N + \text{Nd}_N)/2$, and $\text{Ti}^* = (2\text{Dy}_N + \text{Eu}_N)/3$. Precision ranges from 5% to 10% for Zr and Ti in both clinopyroxene and orthopyroxene. For REE, it is usually better than 10% in clinopyroxene and better than 30% in orthopyroxene. Accuracy is believed to be of the same order as the precision^{1,3}. The propagation of statistic error in the calculated bulk-rock REE abundances is essentially due to the overall uncertainty for clinopyroxene, and it is only marginally affected by that for orthopyroxene. On these grounds, the precision for recalculated bulk compositions was better than $\sim 12\%$. In the worst case, the overall error on measured clinopyroxene and orthopyroxene allows the ratios Ti/Ti^* and Zr/Zr^* calculated for the bulk rocks to vary within $\pm 25\%$.

inferred that Ti and Zr depletions in mantle clinopyroxenes should reflect magmatic fractionation processes occurring in bulk rock. But they did not investigate the possibility that negative anomalies in clinopyroxene might be compensated by different partitioning behaviour in other mantle minerals.

Bulk-rock compositions of HFSE and REE for spinel peridotite xenoliths showing no Ti or Zr anomalies with respect to REEs have been documented¹⁷, thereby contradicting the hypothesis of a widespread HFSE depletion, at least in sub-continental mantle. Jochum *et al.*¹⁷ also showed evidence from one peridotite sample which contained HFSE-depleted clinopyroxene but had a corresponding bulk rock composition without HFSE-depletion. They concluded that the trace element pattern of clinopyroxene does not necessarily reflect that of the whole rock, but they did not discuss possible explanations for this discrepancy. A similar conclusion was reached in refs 18 and 19. Sun² has discussed the implications of the results of Salters and Shimizu¹ on growth mechanisms of the lithospheric mantle.

We analysed REE and HFSE concentrations in coexisting clinopyroxene and orthopyroxene pairs from northern Apennine (Italy) peridotites. *In situ* analyses were done with a CAMECA IMS 4f ion microprobe at the CNR-Centro di Studio per la Cristallografia e la Cristallografia, Pavia. Details of precision and accuracy are given in Table 2, and the analytical procedure is described in refs 20 and 21.

The peridotite samples belong to the ophiolitic sequences outcropping in the External Liguride (EL) units^{22,23}. EL peridotites have previously been interpreted^{24,25} as fragments of sub-continental lithospheric mantle emplaced at shallow levels during early stages of rifting of the Jurassic Ligurian-Piedmontese basin. They consist mainly of lherzolites with considerable concentrations of calcic clinopyroxene (up to 10–15% by volume), and record a complex history of metamorphic re-equilibration from spinel- to plagioclase-facies stability field^{24–27}. In the spinel assemblage, clinopyroxene, orthopyroxene and spinel occur as zoned porphyroclasts, with the cores still retaining sp-facies compositions. Clinopyroxene and orthopyroxene recrystallize in granoblastic aggregates in textural equilibrium with plagioclase, changing their major and trace element abundances (Al_2O_3 , Na_2O and Sr decrease, Cr_2O_3 increases)²⁶. Here we will not discuss chemical variations due to metamorphic re-equilibrations; we

will consider only porphyroclastic cores of clinopyroxene-orthopyroxene pairs stable in the spinel-facies stability field. Two representative samples were investigated in detail. Their bulk rock compositions are reported in Table 1. Within each sample, several coexisting pairs of clinopyroxene and orthopyroxene were analysed. In both samples, the ranges of REE, Ti and Zr contents in clinopyroxene and orthopyroxene are very narrow²⁶; representative analyses are listed in Table 2 and plotted in Fig. 1.

The composition of the peridotites investigated is not very depleted, thus resembling many sub-continental lithospheric peridotites. This is shown by their bulk-rock major element abundances (Table 1) and clinopyroxene REE concentrations (up to 10–15 times those of C1 chondrites²⁸; $\text{Ce}_N/\text{Yb}_N = 0.78$ –1.0; see Fig. 1; Table 2), which closely resemble clinopyroxenes in undepleted sub-continental-type sp-lherzolite xenoliths²⁹ and orogenic peridotites^{30,31}. Orthopyroxenes show very low REE contents (lower than those of C1), which are consistent with clinopyroxene being the main REE carrier in mantle assemblages^{1,14–16,29}. This is not true, however, for Ti and Zr. In fact, although the clinopyroxenes show well-developed Ti and slight Zr depletion ($\text{Ti/Ti}^* = 0.50$ –0.57; $\text{Zr/Zr}^* = 0.72$ –1.01) as noted previously¹, the coexisting orthopyroxenes are characterized by strongly positive Ti and Zr anomalies with respect to the neighbouring REEs ($\text{Ti/Ti}^* =$

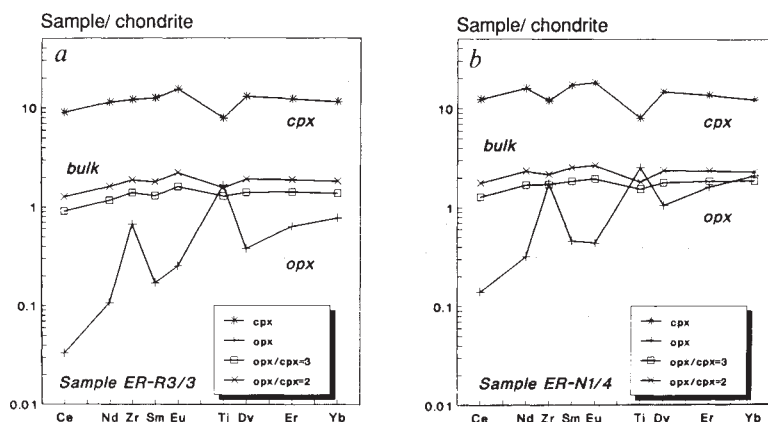


FIG. 1 Chondrite-normalized REE, Ti and Zr concentrations for: orthopyroxene (opx), clinopyroxene (cpx) and bulk rock (bulk) calculated by mass balance (see text). a, sample ER-R3/3; b, sample ER-N1/4. Normalizing values are taken from Anders and Ebihara²⁸.

3–4.9; $Zr/Zr^* = 4.5$ –4.9). Orthopyroxene is thus important in determining the bulk-rock Ti and Zr contents, depending on the modal orthopyroxene and clinopyroxene abundances. Similar results for Ti have been reached by McDonough *et al.*¹⁹, from an investigation (done on mantle xenoliths) of the distribution of Ti and REEs between peridotitic minerals. Our observation is also consistent with recently published solid-liquid partition coefficients³² for REEs, Ti and Zr in mantle orthopyroxene. Experimental data³² indicate that orthopyroxene has partition coefficients for Ti and Zr that are an order of magnitude higher than those of the 'neighbouring' REEs.

To check the effects of such anomalies for the bulk rock compositions, we calculated the modal abundances for the investigated samples by mass balance, using the major element chemistry of whole rocks and porphyroclastic minerals pertaining to spinel assemblage. The sums of squares of residuals are 0.24 and 0.045 for samples ER-R3/3 and ER-N1/4 respectively. Results are given in Table 2. The opx/cpx ratio is estimated at 2.7 in sample ER-R3/3 and 2.6 in sample ER-N1/4. Consequently, we assumed opx/cpx ratios between 2 and 3 as a reliable range, which includes the uncertainty in the calculated mode. Using these two extreme opx/cpx ratios and the opx and cpx concentrations given in Table 2 and Fig. 1, we calculated the bulk rock REE, Ti and Zr abundances by simple mass balance. The calculated whole-rock patterns (Fig. 1) are essentially flat, especially when one considers the uncertainties of the opx/cpx ratio and the uncertainty of the bulk Ti/Ti* and Zr/Zr* resulting from analytical error estimates (see Table 2). They range between one and two times chondritic values, consistent with published REE contents for EL peridotites^{24,25}. It is therefore evident that negative Ti and Zr anomalies in clinopyroxene are well compensated by corresponding positive anomalies in coexisting orthopyroxene. The residual Ti depletion calculated for bulk rocks (Fig. 1) is possibly significant only when using the lower value of the opx/cpx range (opx/cpx = 2: BR1 in Table 2). This is, however, the least reliable ratio. A more precise bulk estimate, including also olivine and spinel, would probably show no Ti depletion at all. Kelemen *et al.*³² note that olivine and spinel, like orthopyroxene, have $D_{sol/liq}$ values for Ti and Zr that are 1–2 orders of magnitude larger than those for the REEs, and these minerals may also help to compensate HFSE depletion in clinopyroxene.

Although our results do not solve the enigma of the lack of equilibrium between HFSE-depleted mantle clinopyroxenes and MORBs with no such depletion, they show that, for melts in equilibrium with a mantle source, the clinopyroxene–melt system is representative with regard to the REEs but not for Ti and Zr, as orthopyroxene is another significant host for these two elements. Exact knowledge of diopside–melt partition coefficients for Ti and Zr relative to the REEs is essential if we are to resolve this contradiction. Johnson *et al.*³ themselves have noted that, although most published diopside–melt partition coefficients do not show negative anomalies of Ti and Zr relative to the neighbouring REEs, the evidence (in clinopyroxene from abyssal peridotites) of increasing Zr depletion with increasing degree of melting indicates that $D_{cpx/liq}$ for Zr is lower than for the adjacent REEs. This strongly suggests that the relevant clinopyroxene–melt partition coefficients have not yet been measured accurately enough. We have not addressed the question of liquid–crystal partition coefficients directly. Rather, by analysing another mineral phase (orthopyroxene) in mantle peridotites, we have demonstrated that negative Ti and Zr anomalies in clinopyroxene from mantle peridotites cannot be used to infer bulk-rock HFSE depletion without data from other major minerals. The Ti and Zr enrichment in orthopyroxene compensates for the depletion in clinopyroxene, and the overall results simply reflect different partitioning of Ti and Zr (relative to REEs) between mantle minerals. Although we have based our case only on two samples from one peridotite occurrence, our results are straightforward, and may be exemplary for other

sub-continental-type peridotites. More complete analysis of both whole-rock compositions and the constituent mineral phases are needed to revive the claim of a worldwide HFSE-depleted mantle. □

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Large-scale eradication of rabies using recombinant vaccinia–rabies vaccine

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RABIES infection of domestic and wild animals is a serious problem throughout the world. The major disease vector in Europe is the red fox (*Vulpes vulpes*) and rabies control has focused on vaccinating and/or culling foxes. Culling has not been effective, and the distribution of live vaccine baits is the only appropriate method for the vaccination of wild foxes¹. Although some European countries have conducted field vaccination campaigns using attenuated rabies virus strains^{2–5}, their use has not been extensively approved because they retain pathogenicity for rodents and can revert to virulence^{6,7}. These strains cannot be used in North America because they are pathogenic for the striped skunk (*Mephitis mephitis*)⁸ and

are ineffective in the racoon (*Procyon lotor*)⁹. We have constructed a recombinant vaccinia virus¹⁰, VVTGgRAB, expressing the surface glycoprotein (G) of rabies virus (ERA strain)^{11–13}. The recombinant was a highly effective vaccine in experimental animals^{11–13}, in captive foxes^{14,15} and in racoons⁹. We report here the results of a large-scale campaign of fox vaccination in a 2,200 km² region of southern Belgium, an area in which rabies is prevalent. After distribution, 81% of foxes inspected were positive for tetracycline, a biomarker included in the vaccine bait and, other than one rabid fox detected close to the periphery of the treated area, no case of rabies, either in foxes or in domestic livestock, has been reported in the area.

The field vaccine-bait system¹⁵ containing VVTGgRAB is safe for laboratory, domestic and European wild animals^{15–23}, and the duration of immunity elicited (more than 12 months in cubs and 18 months in adult foxes) corresponds to the length of protection necessitated by the life expectancy of foxes (1.5–2.5 years)^{16,17}. Small-scale field trials of fox vaccination^{24–26} were therefore conducted in southern Belgium for safety assessment (data not shown). No abnormal morbidity or mortality was recorded in wild animals by hunters and forestry rangers nor in domestic animals by farmers and veterinary practitioners. But the true efficacy of the vaccination procedure could not be evaluated because of the small size of the areas, the minimum size recommended by the WHO for this purpose being 2,000 km² (ref. 27). A 2,200 km² region of southern Belgium (Fig. 1) was approved for full-scale vaccination in September 1989. The 25,000 baits containing VVTGgRAB and a tetracycline biomarker were dropped by helicopter on three occasions (November 1989, April 1990 and October 1990). After each vaccination campaign, foxes found dead (or shot by hunters) were collected for rabies diagnosis and bone tetracycline analysis. The three collection periods (Jan–Mar 1990; Apr–Oct 1990; Nov 1990–Apr 1991) permitted the collection of 10 rabid and 178 healthy foxes. The bait uptake rate determined from tetracycline status in 23 adult foxes (9 rabid, 14 healthy) during the first period was 74%; six of the 9 rabid and 11 of the 14 healthy foxes were positive for tetracycline. During the second collection period bait uptake rates were 80% (25/31) in adult foxes while only 49% (27/55) in juveniles; during spring dispersal of baits most cubs (born principally in March) would be expected to be confined to the immediate surroundings of the breeding den and thus less likely to ingest baits. No rabid fox was recorded during this period (0/86). After the third phase of vaccination (October 1990) 81% (64/79) of inspected animals were tetracycline-positive. The one rabid animal recorded, at the periphery of the baited area, was tetracycline-negative. The geographical distribution of these animals is given in Fig. 2.

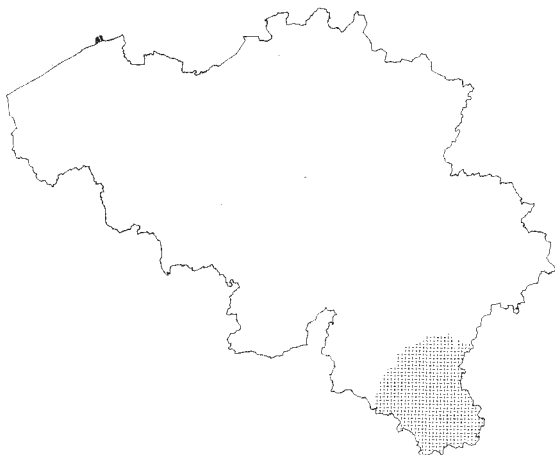


FIG. 1 Target area for large-scale fox vaccination; a 2,200 km² region in the province of Luxembourg (southern Belgium).

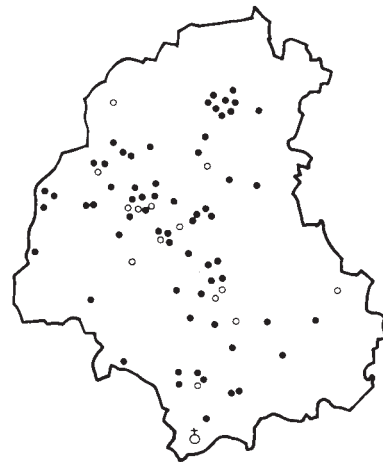


FIG. 2 Geographic distribution of 79 foxes (78 healthy, 1 rabid) shot or found dead in the area vaccinated three times (collection period: October 15, 1990 to April 30, 1991). ●, Animals tetracycline-positive; ○, animals tetracycline-negative; ♀, rabid tetracycline-negative fox.

METHODS. VVTGgRAB propagated on cultured green monkey kidney cells (VERO) was used to establish a freeze-dried master stock from which all vaccine preparations derive (≤ 5 passages). Vaccine-baits¹⁵ were formed from a mixture of plant and animal proteins and fish oil aggregated using a synthetic polymer and contained tetracycline hydrochloride (150 mg per bait) as a biomarker. A sealed plastic sachet containing 2.5 ml liquid vaccine ($\geq 10^8$ TCID₅₀) was introduced into the bait, stitched closed, and baits were stored at 4 °C until distribution. Baits were dropped from helicopter (average height 80 m) according to a grid resulting in a mean density of 15 km⁻²; urban areas were not seeded. Tetracycline was detected in the left jaw of culled animals or animals found dead using ultraviolet fluorescence microscopy of a 400- μ m transverse section (diamond saw, Isomet-Buehler). The presence of brain rabies virus was determined by immunofluorescence and intracerebral inoculation of mice as recommended by the WHO (ref. 31).

Despite the dramatic decrease in the number of rabid foxes recorded after vaccine-bait distribution, the efficacy of the vaccination campaign is difficult to evaluate because systematic collection of foxes is not logistically feasible. But transmission of rabies to domestic animals occurs through the bite of a rabid wild animal. Because notification of cases of rabies in cattle and sheep is mandatory in Belgium the incidence of rabies in domestic livestock provides a reliable indicator of the prevalence of rabies in the wild. Figure 3 plots the number of notified cases of rabies in livestock over a 7-year period before, during and after the vaccination campaigns. No case of livestock rabies has been recorded in the study zone since the second phase of vaccination.

Our results indicate that the dispersion of live recombinant vaccinia virus VVTGgRAB can be an effective means of controlling rabies. But a number of issues remain to be addressed. First, after the first phase of vaccination (but not subsequently) a number of foxes were recorded that were positive for both tetracycline and rabies infection. Because the efficacy of the vaccine-bait system was previously demonstrated in captive foxes^{14,15} when animals resisted severe challenge 30 days post-vaccination, we surmise that the animals may have been incubating rabies at the time of vaccination. Second, the uptake rate of vaccine by cubs (49%) compared with adults (80%) when vaccine dispersal took place in the spring falls below the critical fraction (p) of immunized animals required to prevent spread of rabies. This fraction depends on the mean density of target animals, and for foxes can be estimated from the relation $p \geq 1 - K_T/K$, where K_T is the density of foxes necessary to maintain the endemic persistence of rabies and K is the actual fox density in the absence of rabies^{28,29}. K_T is estimated to be roughly 0.4 foxes per km² and the average K value in Europe is 1 fox per km² (ref. 29), although this figure can vary from 0.1 in poor

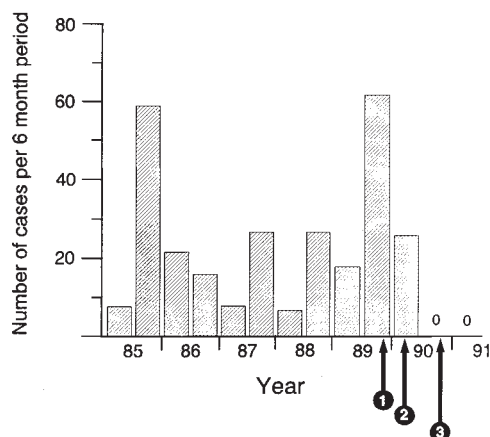


FIG. 3 Seven-year evolution of the incidence of rabies in domestic livestock. Histogram boxes plot the half-yearly (Jan-Jun and Jul-Dec) numbers of cases of rabies notified in sheep and cattle in the target area before and following vaccination. The arrows indicate the rough timing of the three large-scale campaigns of vaccine-bait dispersal.

biotic areas to over 4 in some suburban areas³⁰. Thus, for a K value of 2 foxes per km², a probable overestimation of the mean density in the rural area under study, the proportion of the population (p) which must be vaccinated is 0.8. This indicates that vaccination campaigns might be more effective in the autumn when young foxes forage independently. But our observations indicate that an overall bait uptake rate of 74–81% is sufficient to break the cycle of rabies infection and retransmission in the area under study.

We have also investigated the economics of the vaccine-bait dispersal programme. The average yearly cost of rabies in Belgium (1980–89), including treatment of humans, animal diagnosis, compensation to farmers for the culling of infected livestock, and the culling of wild foxes, is estimated to be 400,000 ECUs 10,000 km⁻², or 88,000 ECUs per annum for the area under study. These figures do not include the cost of vaccination of domestic animals nor the salaries of civil servants. In comparison we estimate the overall expenditure during the three campaigns of vaccine-bait distribution (bait, helicopter and personnel costs) to be 118,000 ECUs. Because vaccination following eradication can, in principle, be interrupted or subsequently limited to the borders of vaccinated zone, long-term maintenance of a rabies-free area by peripheral vaccination with live recombinant vaccinia virus VVTGgRAB may well be economically justifiable. □

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Mouse *Small eye* results from mutations in a paired-like homeobox-containing gene

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SMALL eye (*Sey*) in mouse is a semidominant mutation which in the homozygous condition results in the complete lack of eyes and nasal primordia. On the basis of comparative mapping studies and on phenotypic similarities, *Sey* has been suggested to be homologous to congenital aniridia (lack of iris) in human^{1,2}. A candidate gene for the aniridia (*AN*) locus at 11p13 has been isolated by positional cloning³ and its sequence and that of the mouse homologue has been established (C.T., manuscript in preparation). This gene belongs to the paired-like class of developmental genes first described in *Drosophila* which contain two highly conserved motifs, the paired box and the homeobox^{4,5}. In vertebrates, genes which encode the single paired domain as well as those which express both motifs have been described as the Pax multigene family^{6–10}. A Pax gene recently described as *Pax-6*^{11,12} is identical to the mouse homologue of the candidate aniridia gene. Here we report the analysis of three independent *Sey* alleles and show that indeed this gene is mutated and that the mutations would predictably interrupt gene function.

Several *Sey* alleles have arisen independently, all of which are semidominant^{13,14}. The phenotype found in mutant *Sey* mice is typified by the 15-day post coitum (p.c.) embryos in Fig. 1. The obvious defect in the heterozygous *Sey*/+ embryos (Fig. 1b) is that the eyes are smaller than wild type (Fig. 1a). Often the fissure of the optic cup is delayed in closing and there is folding at the margin of the optic cup resulting in a keyhole shaped opening (arrow in Fig. 1b). In the homozygous *Sey*/*Sey* embryo (Fig. 1c) no eyes are visible and the nasal cavities do not develop^{15,16} resulting in arrowed, imperforate snouts.

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Newborn homozygotes die soon after birth as they cannot breathe while suckling.

We have analysed the *Pax-6* gene in three different alleles of *Small eye* including the original spontaneous derived mutation designated *Sey*^{13,15}, a radiation induced mutation designated *Sey*^H (ref. 14) and an ethylnitrosourea (ENU)-induced mutation designated *Sey*^{Neu}. We consider, first, the radiation-induced allele *Sey*^H. This mutation has more severe effects than the other *Small eye* alleles; the homozygotes die prenatally either before or shortly after implantation¹⁵. The source of the mutational event and the increased severity of the phenotype suggests that the defect is due to a chromosomal deletion. To establish the nature of this mutation, genomic DNA digested with *Taq*I or *Pvu*II was analysed from the parents and offspring of an interspecific mating between heterozygous *Sey*^H/+ females (C57BL/6 background) and male *Mus spretus* mice. The probe used was the 1.6-kilobase (kb) insert in pSM-1³ (Fig. 4), a mouse brain complementary DNA clone corresponding to the AN candidate gene. None of the restriction fragments unique to *Sey*^H/+ parent is detected in the *Sey*^H/*M. spretus* F₁ indicating that the portion of the gene corresponding to the pSM-1 probe is deleted in the *Sey*^H mouse (Fig. 2). As the mouse homologue of the WT-1 (Wilm's tumour) gene is also detected (data not shown), the *Sey*^H deletion covers a large region. Therefore the more severe homozygous phenotype in *Sey*^H is probably due at least in part to the absence of neighbouring genes.

To analyse *Pax-6* in the remaining two *Sey* alleles, total RNA isolated from the heads of 15-day fetal animals was amplified

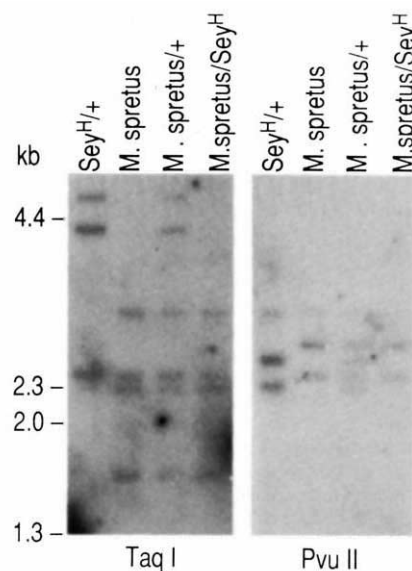


FIG. 2 Deletion analysis of the *Sey*^H allele. Southern blot probed with the *Sey* cDNA pSM-1 containing DNA from the parental types of an interspecific cross between a heterozygous *Sey*^H mutant adult (lanes designated *Sey*^H/+) and wild-type *M. spretus* and resulting offspring showing the wild-type phenotype (*M. spretus*/+) and the mutant phenotype (*M. spretus*/*Sey*^H). The variation in the length of restriction fragments of the parents is most apparent in the DNA digested with the restriction enzymes *Taq*I and *Pvu*II. The bands unique to the *Sey*^H parent do not segregate with the *Sey*^H/*M. spretus* offspring but are present in the wild-type offspring (+/*M. spretus*).

METHODS. The *Sey*^H allele was maintained on the C57BL/6 background for several generations. Male *Sey*^H/+ mice were allowed to freely mate with females from a laboratory strain of *M. spretus*. DNA was isolated from the liver and spleen of all mice analysed by the method of Blin and Stafford³⁵. DNA was digested with restriction enzymes according to manufacturers specification (Boehringer); restriction fragments were separated on 1% agarose gels, blotted and fixed onto Hybond N+ (Amersham) according to the manufacturers details.

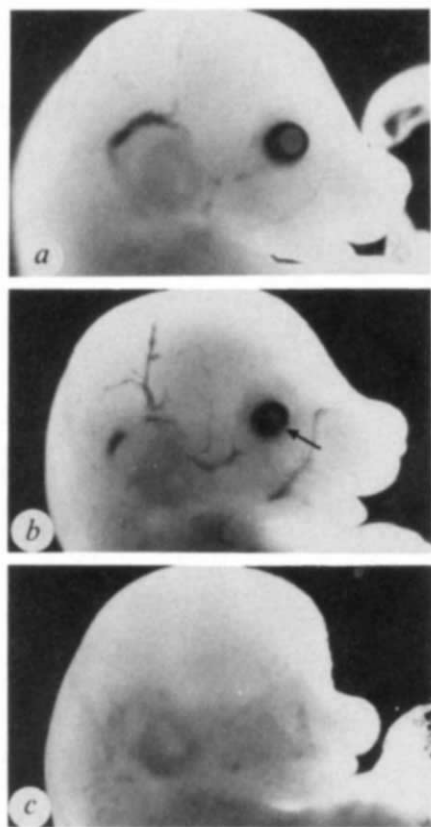


FIG. 1 Phenotype of *Sey* mutant mice. Mouse embryos at 15 days development from a single litter generated from a *Sey*/+ × *Sey*/+ mating showing the features of *a*, wildtype; *b*, *Sey*/+ heterozygote; *c*, *Sey*/*Sey* homozygote. Note in *b* the decrease in the size of the eye, and the keyhole shape region where the optic cup has closed around the lens (arrow). The homozygote *Sey*/*Sey* (*c*) embryo displays no remnants of eye formation at this stage and a much shortened snout. These embryos also appear smaller.

by the polymerase chain reaction (PCR) using two sets of primers which together cover three-quarters of the open reading frame. Primer set A amplified a 384-base pair (bp) fragment encompassing the paired box motif (region A, Fig. 4) whereas set B amplified a 584-bp fragment encompassing the homeobox and flanking regions (region B, Fig. 4).

Possible genetic variations in the original *Sey* allele were defined by the 'HOT' chemical cleavage analysis of DNA mismatches^{17,18}. Heteroduplexes of the PCR fragments generated from *Sey*/*Sey* homozygous and wild-type sibling embryos showed a single base-pair mismatch which occurred in the Region B PCR fragment (data not shown). Subsequent sequence of the PCR products from *Sey* and from wild type showed a base-pair change at codon 194 of the protein resulting from a G:C (in the wild type) to T:A transversion (Fig. 3*a*). This change creates an in frame stop codon converting a GGA triplet encoding glycine to a TGA nonsense codon resulting in a protein terminating before the homeobox (Fig. 4).

A newly established *Sey* allele designated here *Sey*^{Neu} was originally recovered in an ENU mutagenesis experiment and the mutation reported as ENU-2029 (ref. 19). The original mutation carrier expressed a phenotype similar to *Sey* and genetic crosses²⁰ proved the phenotypic variant to be due to an autosomal semidominant mutation. A three point linkage analysis indicated that this mutation maps to chromosome 2 consistent with a location at the *Sey* locus.

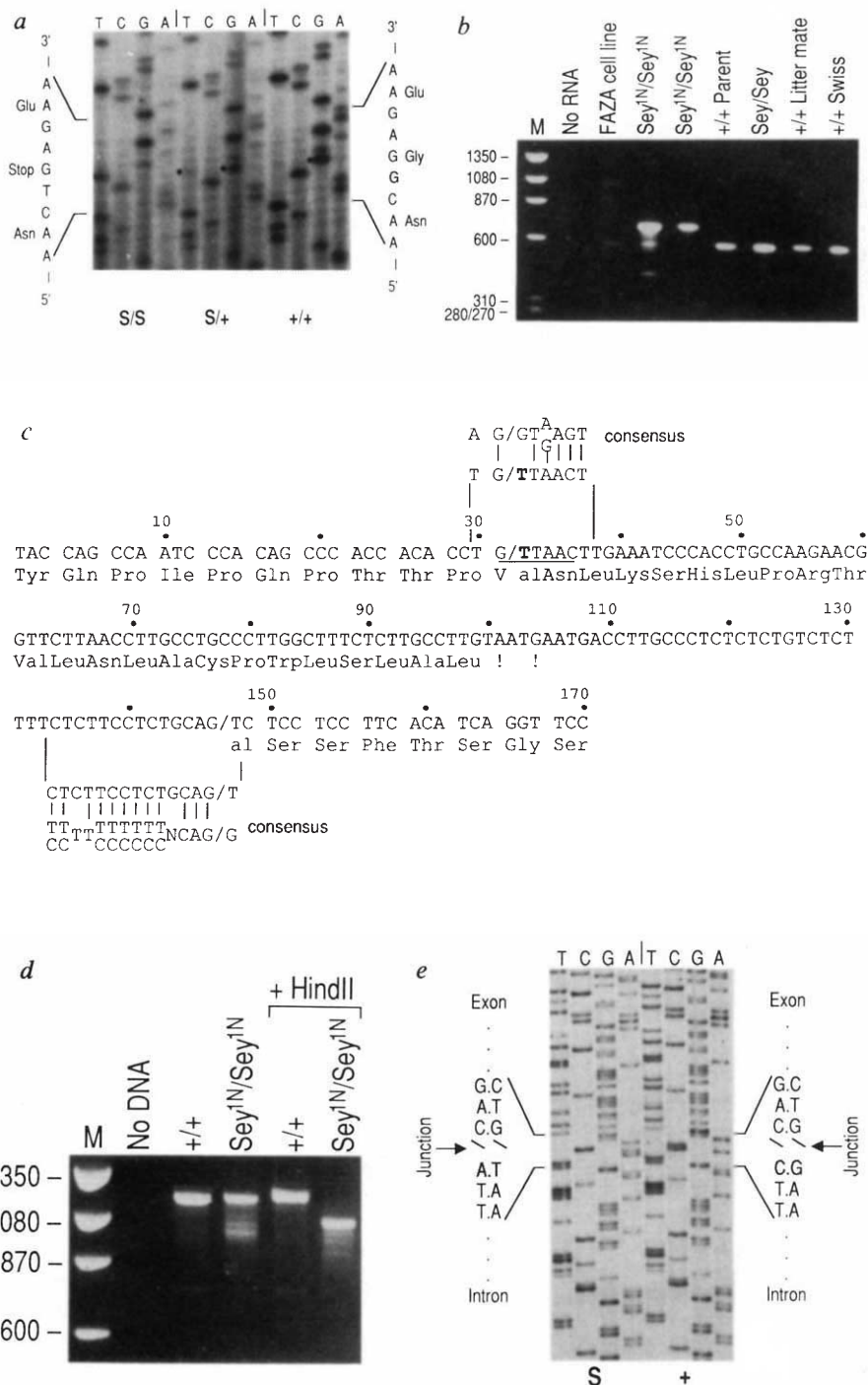
Change in the *Sey*^{Neu} transcript was immediately obvious upon amplification. PCR amplification of mRNA from

Sey^{Neu}/Sey^{Neu} embryos showed an increased fragment length in Region B (Fig. 3b). Sequence analysis of cloned PCR fragments confirmed that the RNA from *Sey^{Neu}* mice contained an additional 116 nucleotides (Fig. 3c) absent from the cDNA sequence at the 3' side of the homeobox. Sequences at the 5' and the 3' ends of the insert compare well with consensus intronic splice donor and acceptor sites²¹. A key exception is at the +1 intronic position (Fig. 3c). This base is invariably a G as shown in a sequence survey of over 3,700 5' splice sites²¹, but the *Sey^{Neu}*

insertion contains a T at this site. To ascertain the origin of the messenger RNA addition, genomic DNA from *Sey^{Neu}* and the wild-type parent was amplified by PCR (Fig. 3d). Fragments of approximately 1.2 kb were generated which were cloned and sequenced. An intron of identical length is found in both *Sey^{Neu}* and wild-type genomic sequences but with a T replacing the wild-type G (Fig. 3e) at the predicted splice site introducing a new restriction site in *Sey^{Neu}* (Fig. 3d). Genetic variants at this intronic site have been found to reduce or abolish splicing²²⁻²⁶

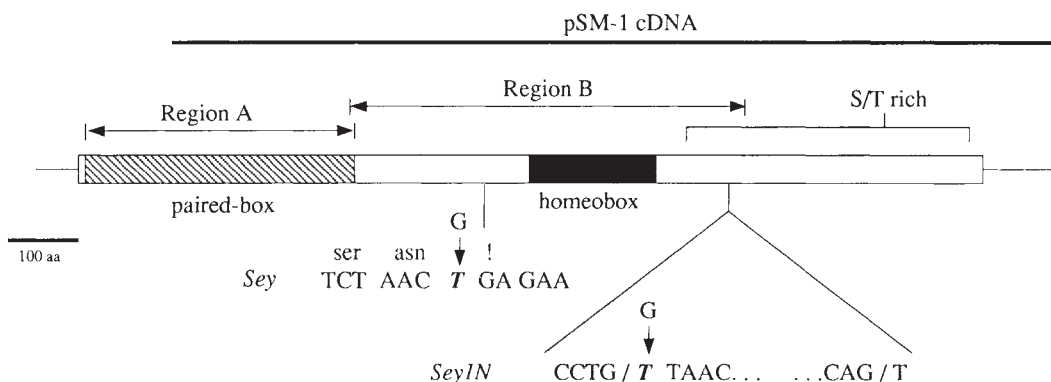
FIG. 3 Analysis of the mutations in the *Sey* and *Sey^{Neu}* alleles. The PCR products generated from RNA from *Sey* (a) and *Sey^{1N}* (b-e) were analysed for genetic change. a, The single base difference detected in *Sey/Sey* embryos by the chemical cleavage mismatch method was analysed by DNA sequencing. The region B PCR fragments (see Fig. 4) from *Sey/Sey* (S/S) homozygotes, *Sey*/(S/+) heterozygotes and wild-type (+/+) were sequenced using an oligonucleotide internal to the fragment. The base pair change of a G to T results in the conversion of a GGA glycine codon (sequencing tracts +/+) to a TGA stop codon (sequencing tracts S/S). The heterozygote (sequencing tracts S/+) shows both a G and a T at this position. The bases of interest are indicated by dots. b, Analysis of the length of the region B PCR fragments generated from *Sey^{Neu}/Sey^{Neu}* animals in comparison to a rat liver cell line (FAZA cell line), (102 × C3H)_{F1} animals on which the mutation was generated (+/+ Parent), *Sey* homozygotes (*Sey/Sey*), wild-type littermates (+/+ Littermate), and outbred Swiss mice (+/+ Swiss). Ethidium bromide stained PCR products were fractionated on a 1.3% agarose gel. c, Sequence of the addition and the surrounding coding region in the transcript from *Sey^{Neu}* animals. The addition begins (at nucleotide 32) and ends (at nucleotide 147) at the region marked by slashes which represent putative splicing junctions. The consensus sequences for the 5' donor splice and the 3' acceptor splice sites are shown matched to the 5' and 3' ends of the addition sequence. The putative amino acid sequence encoded by the addition and the surrounding exon is presented in the three letter code and the position of the predicted stop codons (!) which will truncate the protein are shown. The predicted G to T change is shown as a bold letter at nucleotide 32. This putative nucleotide change would produce a restriction site (*Hind*III) at the splice junction which is underlined. d, PCR fragments generated from genomic DNA isolated from (102 × C3H)_{F1} mice (+/+) and *Sey^{Neu}* mutants (*Sey^{Neu}/Sey^{Neu}*) before and after digestion with the restriction enzyme *Hind*III. e, Sequence analysis of the cloned genomic PCR fragments from *Sey^{Neu}/Sey^{Neu}* (S) and wild type (+) as in d. The G:C to T:A change is highlighted, and the splice junction indicated.

METHODS. Total RNA was isolated from the head of 15-day mouse embryos using the guanidium hydrochloride method³⁶. RNA PCR was done by the method of Kawasaki³⁷ using the cycle time and temperatures suggested. Oligonucleotides used for amplifying region A and B were 22mers (set A-[AGTCACAGCGGAGTGAATCAGC] and [AGCCAGGTTGCGAAGAAGTCTG] and set B-[AACAGAGTTCTTCGCAACCTGG] and [ATGGAACCTGATGTGAAGGAGG]). Direct sequencing of the PCR products was done using oligo509 [TGCCA-GCAACAGGAAGGAGG] which hybridizes internally in the region B PCR fragment and the double-stranded DNA Cycle Sequencing System kit (BRL) according to the manufacturers protocol. The genomic PCR fragments were amplified using oligo509 and the second oligonucleotide of set B using the



conditions of the RNA PCR³⁷ and cloned into Bluescript II SK (Stratagene). Cloned PCR products were sequenced using the Sequenase sequencing kit Version 2.0 (US Biochemical) according to the manufacturers protocol.

FIG. 4 Schematic diagram of the coding region of the mouse *Pax-6* gene. The relative positions of the 384-bp paired box and 180-bp homeobox is shown. The Ser/Thr rich (designated S/T rich) region at the C terminus is bracketed. The position and the nature of the *Sey* and *Sey^{Neu}* mutations are shown below the coding region. The regions amplified by PCR are designated as region A and region B (primers used shown in Fig. 3) and the extent of the pSM-1 cDNA clone is represented by the thick line.



thus accounting for the inhibition of processing of this intron in *Sey^{Neu}*. The predicted mutant protein would contain both the paired box and the homeobox but would lack the 115 amino acids from the C terminus because of an intron-encoded stop codon (Fig. 3c). Although other mechanisms of protein inactivation can be envisaged, the region omitted in the *Sey^{Neu}* protein is composed of a high percentage of serine and threonine residues (25% over a stretch of 135 amino acids) (Fig. 4). Such endowed domains are found in the Oct-1²⁷, and Spl²⁸ transcription factors. Although the function of Ser/Thr domains is unclear, 'domain switching' analysis indicates a role for them in transcriptional activation²⁹.

The *Sey* mutations afford an intriguing link between pattern formation in the face and morphogenetic events in the eye. Studies of the mutant morphology suggest a common element in these developmental events identifying aberrant lens and nasal placode formation as a primary event in this phenotype^{15,16}. The lens placode which gives rise to the lens proper is involved in inducing morphogenesis in the optic cup. In homozygous mutants the lens placode does not develop, presumably resulting in degeneration of the optic cup; whereas in the heterozygote the lens placode and the resulting lens are smaller than in wild type and the cells are disorganized¹⁵. In contrast, the development of the nasal pits is apparently only affected in the homozygote. Our own expression studies (unpublished data and ref. 3) and the more extensive ones of Walther and Gruss¹² are consistent with the gene's having an earlier role in placode formation. The expression of this gene however is quite extensive. A recently described zebrafish *pax* gene, *pax[zfa]*, which by our analysis is the homologue of the *Sey* gene (one amino acid difference comparing the paired and homeoboxes), is expressed in a specific region of the diencephalon and throughout the spinal cord³⁰. Expression in the brain and spinal cord is confirmed in mouse studies¹². Nevertheless, no brain or spinal cord mutant phenotype has been reported in *Sey* mice. Closer examination of derived structures in the mutants may prove of interest.

Interpreting the semidominant nature of these mutations may indicate mechanisms by which this gene operates. The fact that *Sey/+* and *Sey^{Neu}/+* present a similar phenotype which resembles the eye phenotype of the deletion *Sey^H* heterozygote suggests that *Small eye* may be due to reduced dosage of protein activity. If this is the case, it would suggest that the *Pax-6* function is dependent on a defined threshold concentration.

This analysis indicates that *Pax-6* is the mouse *Sey* gene. The developmental importance of the Pax multigene family has been further highlighted by the finding that two other Pax genes are involved in mouse mutations (reviewed in ref. 31), *Pax-1* in

*undulated*³² and *Pax-3* in *Splotch*³³. Are mutations in these genes responsible for human congenital abnormalities? The finding that *Pax-6* is mutated in *Sey*, the condition proposed to be homologous to human aniridia, suggests that Pax genes may be involved in the aetiology of human diseases. *Pax-6* joins two other homeobox-containing genes, *Hox-7* and *Hox-8*, expressed in the eye³⁴ and suggests that genes containing this domain are important in the development and pattern formation of this structure. The molecular analysis of these genes in combination with additional eye mutations may enable a more complete understanding of the intricate details of eye development. □

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Altered chloride ion channel kinetics associated with the $\Delta F508$ cystic fibrosis mutation

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CYSTIC fibrosis is associated with a defect in epithelial chloride ion transport (reviewed in refs 1, 2) which is caused by mutations in a membrane protein called CFTR (cystic fibrosis transmembrane conductance regulator)³. Heterologous expression of CFTR produces cyclicAMP-sensitive Cl^- -channel activity⁴⁻⁷. Deletion of phenylalanine at amino-acid position 508 in CFTR ($\Delta F508$ CFTR) is the most common mutation in cystic fibrosis⁸. It has been proposed that this mutation prevents glycoprotein maturation and its transport to its normal cellular location⁹. We have expressed both CFTR and $\Delta F508$ CFTR in Vero cells using recombinant vaccinia virus. Although far less $\Delta F508$ CFTR reached the plasma membrane than normal CFTR, sufficient $\Delta F508$ CFTR was expressed at the plasma membrane to permit functional analysis. $\Delta F508$ CFTR expression induced a reduced activity of the cAMP-activated Cl^- channel, with conductance, anion selectivity and open-time kinetics similar to those of CFTR, but with much greater closed times, resulting in a large decrease of open probability. The $\Delta F508$ mutation thus seems to have two major consequences, an abnormal translocation of the CFTR protein which limits membrane insertion, and an abnormal function in mediating Cl^- transport.

The CFTR protein was expressed using a highly reproducible, complementary vaccinia virus (VV) coexpression system¹⁰, in which a first virus expresses constitutively the phage T7 RNA polymerase under control of the VV7.5K promoter (VVTG1193), and a second virus expresses the coding part of the CFTR complementary DNA or its $\Delta F508$ mutant form under control of the T7 promoter (VVTG5959 and VVTG5968, respectively; Fig. 1a). Coinfection of Vero cells, a monkey kidney fibroblast cell line, with the respective complementary viruses, resulted in the specific expression of a protein of relative molecular mass 140,000 (M_r 140K) representing the non-mature form of CFTR^{9,11} and also of a more diffusely migrating band corresponding to the mature glycosylated 170K species of CFTR^{9,11}, which were both identified after metabolic labelling and immunoprecipitation (Fig. 1c). Cell-surface radioiodination with ¹²⁵I by lactoperoxidase followed by a specific immunoprecipitation revealed only the 170K species (Fig. 1d), suggesting that this is the form of CFTR in the plasma membrane. Although the 140K form was expressed in equal amounts for both CFTR and $\Delta F508$ CFTR (Fig. 1b, c), western blot analysis (Fig. 1b) shows that only a minor proportion of the $\Delta F508$ CFTR protein was processed to the 170K form, consistent with other results⁹. This proportion was too small to identify $\Delta F508$ CFTR clearly at the cell surface by labelling with lactoperoxidase (Fig. 1d). Our tentative conclusion is that CFTR is present in the plasma membrane in its 170K form, and the conversion of the protein from 140 to 170K is severely altered by the $\Delta F508$ mutation.

Identified differences in the presence of CFTR and $\Delta F508$

CFTR at the cell surface may not be quantitative (structural differences between normal and mutant might mean different reactivity towards labelling agents). Therefore an analysis of the comparative anion transport activities of CFTR and $\Delta F508$ CFTR was essential and was first measured by ¹²⁵I efflux¹². cAMP-activated ¹²⁵I efflux was observed in CFTR-expressing cells (Fig. 2a). Although no 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (cpt-cAMP) effect was observed in mock infected cells, a slow stimulation was observed in $\Delta F508$ CFTR-expressing cells (for efflux times of more than 3.5 min, results were statistically different from control; $P < 0.0001$), but the maximal level of this stimulation was systematically lower than in CFTR-expressing cells (Fig. 2b). Ionomycin had no effect on control Vero cells.

The whole-cell patch-clamp shows that in 9 out of 14 CFTR-expressing cells, cAMP induced a rapid and sustained increase in whole-cell currents (about 10-fold) (Fig. 2d). Because Cl^- was the only conducting ion, current changes could be attributed to changes in Cl^- conductance. The current voltage ($I-V$) relationship of the cAMP-activated current was linear, with no voltage-dependence as there is for cAMP-induced Cl^- currents in epithelial colonic cells¹³ and in other cells expressing heterologous CFTR^{4,6,7}. In three out of four $\Delta F508$ CFTR-expressing cells, a smaller increase in whole-cell current was also recorded after cAMP stimulation (two- to threefold; Fig. 2f), with the same $I-V$ relationship as for CFTR-expressing cells. Control cells never responded to cAMP ($n = 5$) (Fig. 2h). These Cl^- channels were insensitive to 5-nitro-2(3-phenylpropylamino)benzoate (NPPB) (50 μ M) which blocks other types of epithelial Cl^- channels¹⁴.

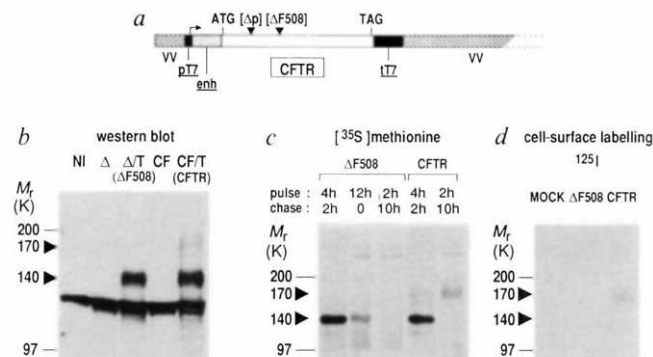


FIG. 1 Expression of CFTR and $\Delta F508$ CFTR by VVTG5959 and VVTG5968, respectively. a, Schematic representation of VVTG5959 and VVTG5968. VV, Vaccinia sequences; pT7, T7, promoter and terminator for the phage T7 RNA polymerase; enh, encephalomyocarditis virus translation enhancer; Δp , mutation of the cryptic promoter (only in VVTG5968); $\Delta F508$, deletion of the residue 508. b, Western blot analysis of CFTR and $\Delta F508$ CFTR with a monoclonal antibody (MATG1031) raised against the 107–117 sequence of CFTR. NI, Noninfected; Δ , Δ /T, infection with VVTG5968 alone or together with VVTG1193; CF, CF/T, idem for VVTG5959. c, Metabolic labelling and immunoprecipitation of CFTR from VV-infected Vero cells. d, Surface labelling and immunoprecipitation of CFTR from VV-infected Vero cells (mock, wild-type VV).

METHODS. a, The coding portion of the CFTR cDNA was isolated and reconstituted from human lung messenger RNA by classic recombinant DNA methods. In the $\Delta F508$ CFTR version of the cDNA, the cryptic bacterial promoter was inactivated by site-directed mutagenesis. Both cDNAs were subcloned in the *Sma*I site of pTG6111 and recombinant VV were obtained by homologous recombination with the VV genome. b, Vero cells were coinfecting with 5 plaque-forming units (p.f.u.) VVTG1193 and 5 p.f.u. VVTG5959 or 5968. Controls were either noninfected cells or cells infected with 5 p.f.u. VVTG5959 or 5968 alone. Western blots were developed with the ECL system (Amersham). c, VV-infected cells were [³⁵S]methionine-labelled (16 to 24 h postinfection). Controls were either noninfected cells or cells infected with 5 p.f.u. VVTG1193 alone. Immunoprecipitation and electrophoreses were done as in ref. 21. The rabbit antiserum used at a 100-fold dilution was raised against the R domain³ (sequence 588–830) of CFTR. d, Intact cells were labelled with ¹²⁵I by lactoperoxidase as in ref. 22 and immunoprecipitated as in b.

Single-channel activity was detected in both CFTR- and Δ F508 CFTR-expressing cells (Fig. 3a). The open probability did not vary with the applied voltage (Fig. 4a). $I-V$ relationships (Fig. 3b) were roughly linear with a slope conductance of 4.9 pS for CFTR-expressing cells, and 4.3 pS for Δ F508 CFTR. The reversal potential was close to 0 mV in both cases. These values were the same with sodium chloride (not shown) or *N*-methyl-D-glucamine chloride in the pipette, demonstrating that the channel is a Cl^- channel. The anion permeability sequence measured as previously described¹⁵ was identical for CFTR- and Δ F508 CFTR-infected cells: $\text{Br}^- > \text{Cl}^- > \text{I}^-$, with $1.5 \leq P_{\text{Br}^-}/P_{\text{Cl}^-} \leq 1.9$ ($n=8$) and $0.3 \leq P_{\text{I}^-}/P_{\text{Cl}^-} \leq 0.5$ ($n=5$). This activity was observed in the presence or absence of the cAMP-elevating cocktail for both CFTR- and Δ F508 CFTR-expressing cells (Fig. 3c). But the intracellular elevation of cAMP drastically increased

the percentage of active patches in both cases (Fig. 3c) suggesting that this channel is responsible for the cAMP-induced increase of ^{125}I efflux and in macroscopic current (Fig. 2). Similar low conductance cAMP-activated Cl^- channels have been described in epithelial cells from pancreatic duct¹⁵, thyroid¹⁶ and colonic cells¹⁷, and recently in cells expressing heterologous CFTR⁷. Although the basic conductive properties were identical in both types (CFTR and Δ F508 CFTR) of infected cells, the mean number of active channels for active patches was smaller in Δ F508 CFTR (1.5 ± 0.8 , $n=30$) than in CFTR expressing cells (6.07 ± 4.1 , $n=42$) ($P=0.0001$). This difference probably explains why mature Δ F508 CFTR was not easily detected in Fig. 1. A nonselective cationic channel was sometimes observed, particularly after a long period of infection, 20–24 h. This channel seems to be endogenous to Vero cells.

Large differences were observed in kinetic properties of the CFTR and Δ F508 CFTR cAMP-stimulated Cl^- channels (for an example, see Fig. 4b). Δ F508 CFTR Cl^- channels remained closed for long periods (minutes), whereas CFTR Cl^- channels tended to have a sustained activity. Thus the open probability was three- to fourfold lower for the Δ F508 CFTR Cl^- channels (Fig. 4a). This difference was due to an increase in closed times for Δ F508 Cl^- channels. No difference was observed in open times (Fig. 4c). Mean open times at -60 mV were very similar for CFTR- and Δ F508 CFTR-expressing cells (2,197 ms ($n=5$) and 1,741 ms ($n=11$), respectively). Open-time histograms gave identical time constants (Fig. 4c). By contrast, mean closed times measured at -60 mV were fivefold higher for Δ F508 CFTR Cl^- channels (17,855 ms, $n=11$) than for CFTR Cl^- channels (3,640 ms) and closed time constants (Fig. 4c) were nearly fourfold higher for Δ F508 CFTR Cl^- channels.

Assuming that the properties of CFTR and Δ F508 CFTR in vaccinia virus-infected cells can be extrapolated to cell affected *in vivo*, our results suggest that the Δ F508 mutation results in:

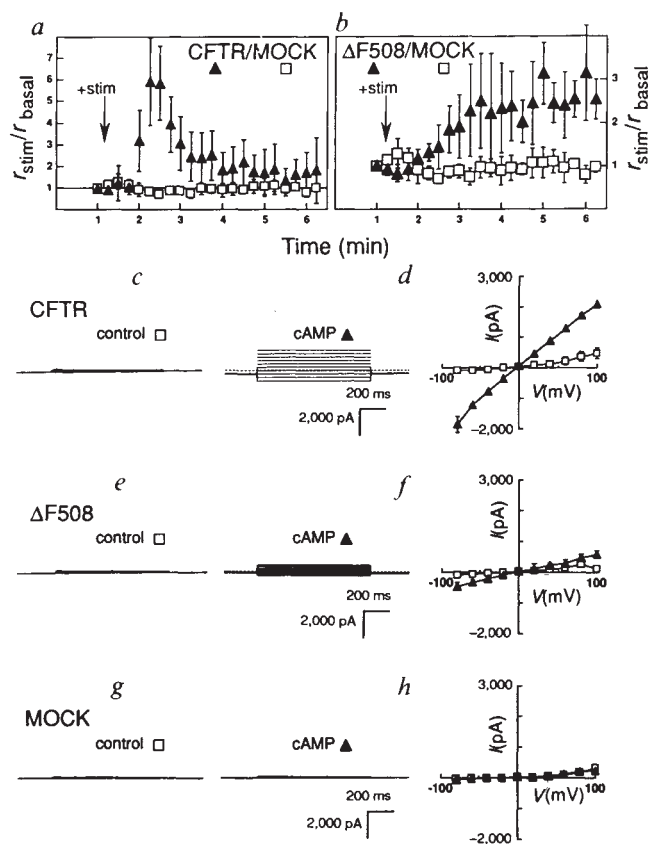


FIG. 2 Functional analysis of CFTR- and Δ F508 CFTR-expressing Vero cells. cAMP-stimulated iodide efflux measured as in ref. 12 in CFTR and mock (a), and Δ F508 CFTR and mock expressing cells (b). Mean I/V relationships for CFTR ($n=2$) (d), Δ F508 CFTR ($n=3$) (f) and mock infected cells ($n=4$) (h) before and after perfusion with the cAMP-elevating cocktail. c, e, g, Corresponding current traces. The probability for $I-V$ relationships for Δ F508 CFTR and mock infected cells to be identical is less than 0.001.

METHODS. a, b, cpt-cAMP (0.4 mM) was added 1.3 min after the end of ^{125}I loading. Results are the mean $\pm$ s.d. of three, six and six experiments for mock, Δ F508 CFTR and CFTR, respectively. The ratio $r_{\text{stim}}/r_{\text{basal}}$ corresponds to the ratio of the efflux rate measured under stimulation conditions by the efflux rate measured under basal conditions, on two different dishes infected for the same length of time. Scale of y axes are different in a and b. d, f, h, Currents were measured at room temperature using the whole-cell patch-clamp technique²³ before and after perfusion with the cAMP-elevating cocktail containing 0.5 mM cpt-cAMP, 10 μM 3-isobutyl-1-methylxanthine (IBMX), 10 μM forskolin. The pipette solution contained in mM: 130 *N*-methyl-D-glucamine chloride (NMDG-Cl), 1 MgCl_2 , 1 CaCl_2 , 2 EGTA/NaOH (100 nM free Ca^{2+}), 2 Na_2ATP , 10 HEPES (pH 7.2). The cell was perfused with (in mM): 140 NMDG-Cl, 1 MgCl_2 , 1 CaCl_2 , 10 HEPES (pH 7.2). To determine $I-V$ relationships, the membrane potential was held at -40 mV and stepped to levels between -80 mV and $+100$ mV in 20 mV increments.

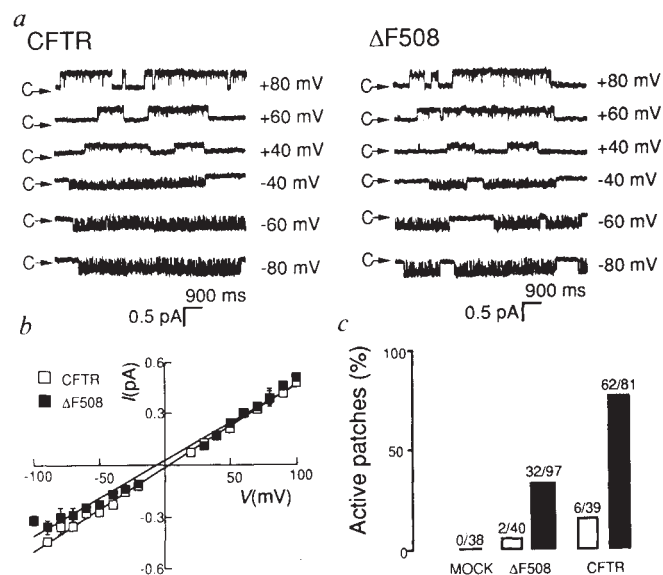


FIG. 3 Cl^- channel activity in CFTR and Δ F508 CFTR-expressing cells. a, Cell-attached recordings on stimulated CFTR- and Δ F508 CFTR-expressing cells (C, closed state). b, Mean $I-V$ relationships (fitted by linear regression) from 12 different CFTR cells and from 9 Δ F508 CFTR cells. c, Numbers of active patches in unstimulated and cAMP-stimulated infected cells.

METHODS. The sign of the applied voltage refers to the bath with respect to the patch pipette. Outward currents refer to the flow of cations from the cell into the pipette. The pipette solution contained (in mM): 140 NMDG-Cl (or 140 NaCl), 1 CaCl_2 , 1 MgCl_2 , 10 HEPES (pH 7.2); bath solution was (in mM): 140 NaCl, 5 KCl, 1 CaCl_2 , 1 MgCl_2 , 10 HEPES (pH 7.2), at room temperature. Methods used for studying relative anion selectivity and for calculating permeability ratios are described in ref. 15.

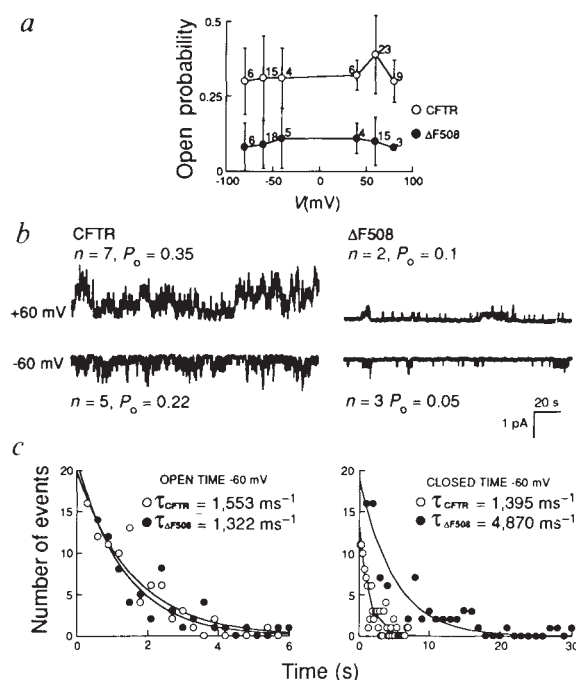


FIG. 4 Comparative kinetics and open probability properties of CFTR and $\Delta F508$ CFTR channels after cAMP stimulation. **a**, Open probability (measured as in ref. 16) at different pipette potentials. (Number of experiments indicated above each point.) The open probability was calculated from patches containing one to seven channels during a period of stable activity as in ref. 16 by assuming that the number of observable unitary current levels during this period represents the number of active channels in the patch. **b**, Typical long-lasting cell-attached recordings. The numbers of active channels (n) and open probability (P_o) are indicated. Sometimes, as already observed⁴⁷, the activity of Cl^- channels slowly declined during long periods of cell-attached recordings. **c**, Histograms of open and closed times (calculated with P-clamp software) obtained from patches containing one single channel from five CFTR- (total recording time of 9 min) and 11 $\Delta F508$ CFTR- (total recording time of 20 min) expressing cells after cAMP stimulation.

a defective maturation of the mutated CFTR, also suggested by Cheng *et al.*⁹, and a functional defect of the $\Delta F508$ CFTR Cl^- channel due to drastically altered kinetics. If 'normal' cystic fibrosis cells behave as Vero cells expressing $\Delta F508$ CFTR, the discovery of specific Cl^- channel activators could lead to a recovery of some significant Cl^- transport activity in patients. Such types of channel activators are already known for voltage-sensitive Na^+ and Ca^{2+} channels^{18,19} and for K^+ channels²⁰.

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Restoration of antigen presentation to the mutant cell line RMA-S by an MHC-linked transporter

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IN mammalian cells, short peptides derived from intracellular proteins are displayed on the cell membrane associated with class I molecules of the major histocompatibility complex (MHC). The surface presentation of class I-peptide complexes presumably alerts the immune system to intracellular viral protein synthesis. Peptides derived from the cytosol must reach the cisternae of the endoplasmic reticulum where they are required for the assembly of stable class I molecules^{1–11}, and it has been proposed that the products of the two MHC-encoded ATP-binding cassette (ABC) transporter genes^{12–15} function to deliver the peptides across the membrane of the endoplasmic reticulum. This idea is supported by experiments in which transfection of a human cell line defective in class I expression with a complementary DNA of one of these genes restored cell surface expression levels¹⁶. Here we show that the complete phenotype of the mouse mutant cell line RMA-S, in which lack of surface expression of stable class I molecules correlates with an inability to present viral peptides originating in the cytosol^{6–10,17}, is repaired by the cDNA of the other transporter gene. These results are consistent with the possibility that the two transporter polypeptides form a heterodimer.

The class II region of the MHC contains two closely linked ABC transporter genes, known in the rat as *mtp1* and *mtp2* (ref. 13); *mtp1*, and its human and mouse homologues, RING4 or Y3 and HAM1 respectively, have been described^{12–15}. Figure 1a shows the sequence of a putatively full-length cDNA clone, 441–11, from the rat *mtp2* gene. The predicted *mtp2* protein is virtually colinear with that of *mtp1*. The dotplot comparison between the two transporter polypeptides (Fig. 1b) shows that each is the other's closest known relative in the evolutionarily ancient ABC transporter family, a similarity emphasized by the hydrophobicity plots (Fig. 1c).

A defect in the expression of the human *mtp1* homologue, Y3, is responsible for a lack of membrane HLA class I expression on the mutant cell line 721.134 (ref. 16). We looked for a similar defect in RMA-S in expression of the mouse homologues of rat *mtp1* and *mtp2* (HAM1 and HAM2 respectively), using rabbit antisera prepared against synthetic peptides based on the predicted *mtp1* and *mtp2* polypeptides. Figure 1d shows that these two antisera detected prominent bands at positions corresponding to a relative molecular mass of about 65,000 (65K) in western blots of reduced Nonidet-P40 lysates of the wild-type RMA cell line. But bands that were apparently identical were seen in lysates from RMA-S. As RMA-S was mutagenized with ethyl methane sulphonate¹⁸, which favours point substitutions¹⁹, it

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was possible that one or other of the transporter polypeptides was present in RMA-S in a functionally inactive form. We therefore transfected RMA-S with full-length cDNA clones encoding the rat MHC-linked ABC transporter genes *mtp1* and *mtp2*. Transfection with *mtp1* failed to produce any significant increase in class I expression (data not shown), but transfection with *mtp2* alone produced an essentially complete restoration of surface class I expression on RMA-S. Fluorescence-activated cell sorter (FACS) analysis of bulk cultures (termed RMA-S.mtp2) using 'conformation-dependent' monoclonal antibodies specific for the $\alpha 1$ and $\alpha 2$ domains of K^b and D^b indicated the presence of high levels of properly folded K^b and D^b class I molecules on the cell surface of the transfectants (Fig. 2).

The failure of class I molecules to assemble with endogenous peptides in RMA-S results in the retention of loosely associated heavy chain/ β_2 -microglobulin complexes in the endoplasmic reticulum or *cis*-Golgi^{17,20}. This retention can be observed in pulse-chase experiments as a failure of the heavy chain to show an increase in molecular mass, which indicates that oligosaccharide side chains are being processed in the Golgi. The pulse-chase profile of D^b in RMA cells showed substantial processing of the molecules during a three-hour chase (Fig. 3a). In RMA-S cells, however, no processing was observed and the heavy chains

were not found associated with β_2 -microglobulin^{6,7,20}. In RMA-S.mtp2 transfectant cells, the kinetics of D^b class I heavy-chain processing were increased almost to those of wild-type RMA and the majority of heavy chains co-precipitated with β_2 -microglobulin, effects comparable to those observed after the addition of exogenous peptides to RMA-S *in vitro*^{6,7}. Similarly, the kinetics of K^b processing were slow in RMA-S cells, whereas those in RMA-S.mtp2 cells were indistinguishable from RMA (Fig. 3b).

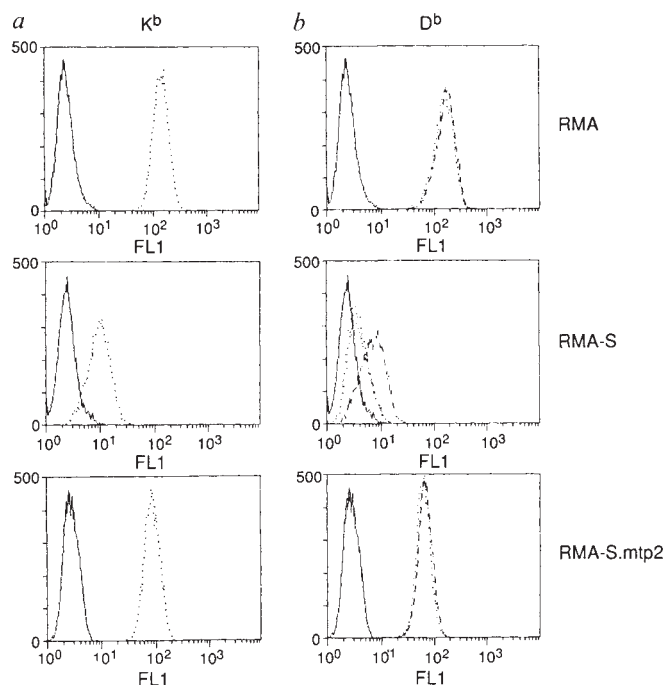


FIG. 2 FACS analysis of cell surface K^b (a) and D^b (b) class I expression on RMA, RMA-S and RMA-S.mtp2 cells demonstrating the increase of both K^b and D^b on RMA-S.mtp2 cells. The monoclonal antibody used to detect K^b was Y-3, specific for the $\alpha 1$ and $\alpha 2$ domains^{6,25}. The antibodies used to detect D^b were B22.249 (---), specific for the $\alpha 1$ domain^{6,7}, 27-11-13S (---), specific for the $\alpha 2$ domain⁷, and 28-14-8S (---), a conformation-independent antibody specific for the $\alpha 3$ domain^{6,7}. Second-stage staining with fluorescein isothiocyanate anti-mouse IgG alone is indicated in each panel by a solid line.

METHODS. *mtp2* cDNA clone 441-11 was cloned into the *Eco*R1 site of the expression vector pH β Apr-1-neo (ref. 26). RMA-S cells were electroporated with *Nde*I-linearized DNA at 160V, 960 μ F using a Gene-pulser with capacitance extender (Bio-rad). After 24 h, selection was initiated in 1.5 mg ml⁻¹ G418 (Gibco-BRL). After 5 days the G418 concentration was reduced to 1 mg ml⁻¹. For FACS analysis, 5×10^5 cells were incubated on ice with the relevant monoclonal antibodies at 5 μ g ml⁻¹. Cells were washed, stained with fluorescein isothiocyanate anti-mouse IgG (Dako), fixed in 1% formaldehyde and analysed on a FACScan (Becton-Dickinson) using Consort 30 software. Ten thousand events were collected. The vertical axis represents relative cell number and the horizontal axis, log of the fluorescence.

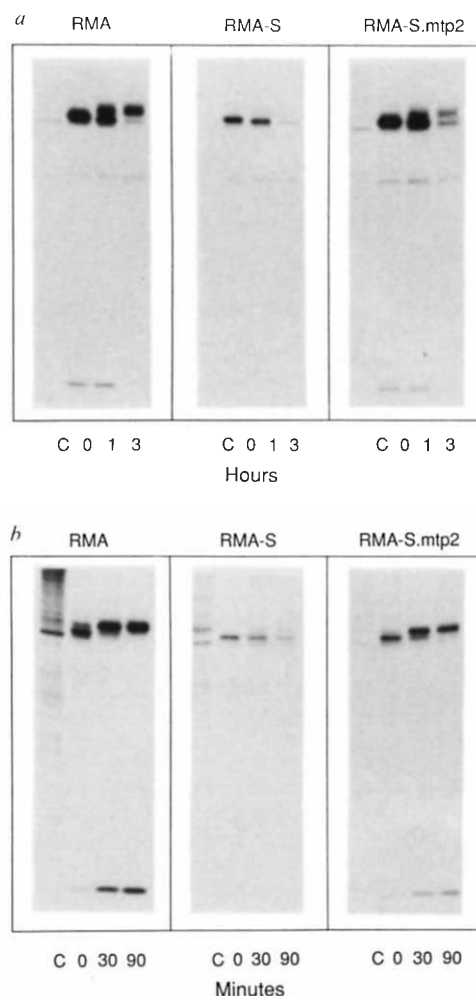


FIG. 3 Pulse-chase analysis of D^b (a) and K^b (b) in RMA, RMA-S and RMA-S.mtp2 cells. D^b was immunoprecipitated with the $\alpha 3$ -domain-specific monoclonal antibody 28-14-8S, and K^b with R1-21.2 (ref. 27). D^b and K^b molecules undergo processing during intracellular transport to the cell surface in both RMA and RMA-S.mtp2 cells. In RMA-S cells stable, processed D^b molecules fail to accumulate during the 3-h chase period (note the absence of β_2 -microglobulin), and K^b is processed very slowly compared with wild-type RMA. Control tracks (C) indicate immunoprecipitation in the absence of anti-class I antibodies.

METHODS. Pulse-chases were performed essentially as previously described²⁴. Cells were labelled with ³⁵S-methionine (ICN-Flow) for 20 min in a and 15 min in b. Incorporation was terminated by the addition of a 20-fold excess of unlabelled methionine. Aliquots of cells were removed at the indicated timepoints and lysed as described²⁴. For the immunoprecipitation of D^b , lysates were precleared for 1 h with protein A-Sepharose (Pharmacia), then D^b was isolated by the addition of 28-14-8S and further protein A-Sepharose for 1 h. The immunoadsorbent was then washed three times and boiled in reducing sample buffer. For immunoprecipitation of K^b , lysates were precleared with sheep anti-rat IgG-Sepharose, then K^b isolated by the addition of R1-21.2 and sheep anti-rat IgG-Sepharose. The immunoadsorbents were washed and processed as above. Samples were analysed by SDS-PAGE on 13% gels, treated with Amplify (Amersham), and fluorographed at -70 °C.

We next asked whether the capacity for antigen presentation had been restored, and if the presence of a transporter polypeptide from another (albeit closely related) species would result in the presentation of the same antigenic peptides. Therefore RMA, RMA-S and RMA-S.mtp2 cells were infected with influenza virus and tested for their ability to be killed by a D^b-restricted cytotoxic T cell line recognizing a nucleoprotein epitope (NP 366–379; refs 17 and 21). As shown in Fig. 4, even after prolonged infection with influenza virus RMA-S cells were lysed poorly, indicating an inability to present antigen efficiently, whereas the RMA-S.mtp2 cells were killed with an efficiency approaching that of control RMA cells. Therefore the correct peptide epitope is made available for binding by D^b in RMA-S.mtp2 cells.

As the lesion in RMA-S cells can be restored to normal, at least qualitatively, by *mtp2* alone, it is likely that the underlying defect in RMA-S lies in the mouse *mtp2* homologue, *HAM2*. Furthermore, fusion of RMA-S cells with the human mutant cell line 721.174, which carries a large deletion in the class II MHC region encompassing both transporter genes¹⁵, fails to complement the defect in RMA-S (data not shown), again implicating this region of the MHC. In conjunction with earlier data¹⁶, our results show that the integrity of both transporter polypeptides is required for any significant class I membrane expression or cytosolic peptide presentation. This conclusion is consistent with a model in which the two transporter polypeptides are required to associate in order to form a functional heterodimer^{16,22}.

The probable existence of a full-length HAM2 polypeptide in RMA-S (Fig. 1d) suggests that the defect may lie in a point mutation in the HAM2 sequence with a large functional effect. The location and identity of this mutation may therefore be of some interest in the analysis of how the MHC-linked transporter genes operate. If the hypothetically defective HAM2 polypeptide in RMA-S participates normally in dimer formation, this

may provide an explanation for the residual quantitative defect in RMA-S.mtp2 transfectant cells compared with wild-type RMA: rat *mtp2* polypeptides may not compete equally with HAM2 for association with HAM1, or otherwise HAM1 may be limiting.

Although the phenotype of RMA-S initially suggested a defect in peptide transport from the cytosol to the endoplasmic reticulum¹⁷, and although that defect can be restored by a polypeptide whose sequence is clearly that of a transmembrane transporter protein, it is important to emphasize that there is no direct evidence that the MHC-linked transporter proteins transport peptides. Much further experimentation is required to establish the role that transporters fulfil in the assembly of class I-peptide complexes. □

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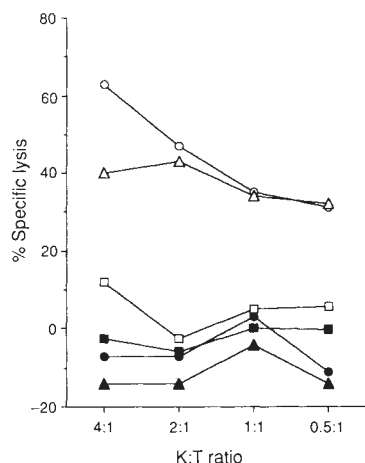


FIG. 4 Killing of RMA and RMA-S.mtp2 cells by influenza virus-specific CTL clone F5. Target cells were: RMA uninfected (●); RMA-S uninfected (■); RMA-S.mtp2 uninfected (▲); RMA infected with influenza E61-13-H17 (○); RMA-S infected with influenza E61-13-H17 (□); and RMA-S.mtp2 infected with influenza E61-13-H17 (△).

METHODS. CTL clone F5, specific for an influenza nucleoprotein epitope encompassed by NP366–379 in association with D^b was tested by ⁵¹Cr-release assay for its ability to lyse influenza E61-13-H17 virus-infected cells as previously described^{17,21}. Sodium 51-chromate-labelled targets (2×10^4), infected for 5.5 h with influenza virus, were exposed to varying numbers of clone F5 cells to generate the ratios of killer to target cells (K:T) shown. After 4 h supernatants were counted for ⁵¹Cr and the per cent specific lysis of targets calculated as (release by CTL-spontaneous release)/(2.5% Triton X-100 release – spontaneous release) $\times 100$.

Ets-related protein Elk-1 is homologous to the *c-fos* regulatory factor p62^{TCF}

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A KEY event in the response of cells to proliferative signals is the rapid, transient induction of the *c-fos* proto-oncogene, which is mediated through the serum response element (SRE) in the *fos* promoter^{1–4}. Genomic footprinting^{5,6} and transfection experiments^{7–9} suggest that this activation occurs through a ternary complex that includes the serum response factor (SRF)¹⁰ and the ternary complex factor p62 (ref. 7). Interaction of p62^{TCF} with the SRF–SRE binary complex requires a CAGGA tract immediately upstream of the SRE (ref. 7). Proteins of the *ets* proto-oncogene family bind to similar sequences^{11–13} and we have found that a member of this family, Elk-1 (ref. 14), forms SRF-dependent

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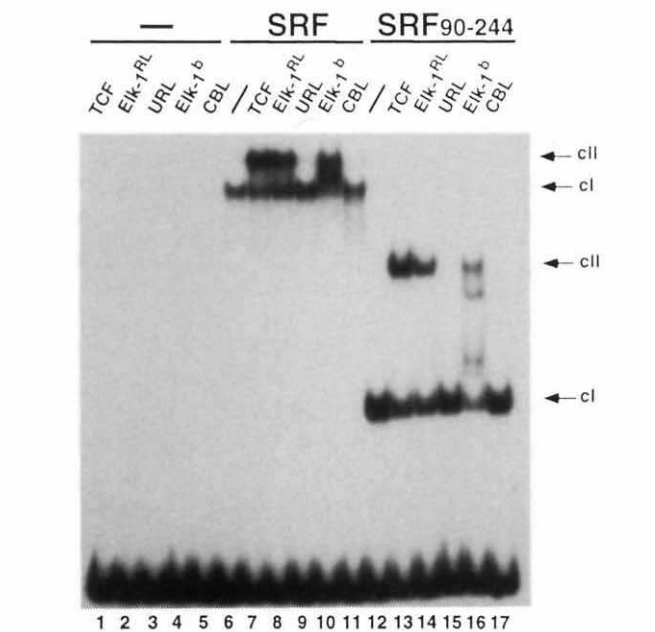


FIG. 1 Elk-1 is a ternary complex factor similar to p62^{TCF}. Gel retardation assay for ternary complex formation by p62^{TCF} and Elk-1. Binding reactions contained, as indicated, p62^{TCF} (TCF), Elk-1 produced *in vitro* (Elk-1^{RL}), unprogrammed reticulocyte lysate (URL), Elk-1 expressed in bacteria (Elk-1^b), or control bacterial lysate (CBL). Additionally, SRF or a truncated version containing amino acids 90 to 244 was added alone (lanes 6 and 12) or together with the proteins exposed to a probe spanning the human *c-fos* SRE. cI is the SRF-SRE binary complex and cII indicates the ternary complex. METHODS. p62^{TCF} purification has been described¹⁸. Elk-1 RNA was synthesized with SP6 RNA polymerase from a cDNA clone linearized with *Bam*HI (ref. 14) and translated in nuclease-treated reticulocyte lysate (Promega). Elk-1 was also expressed in *Escherichia coli* strain BL21 (ref. 12). SRF and SRF₉₀₋₂₄₄ were produced using recombinant vaccinia virus (R.A.H. *et al.*, manuscript in preparation). The probe arises from a subclone of the *c-fos* SRE, positions -331 to -277, and was labelled using polynucleotide kinase. Identical results were obtained with either poly (dI-dC) at 0.23 mg ml⁻¹ or salmon sperm DNA at 0.12 mg ml⁻¹. After incubation at room temperature, complexes were separated by electrophoresis on 4% polyacrylamide gels and visualized by autoradiography of the dried gels⁷.

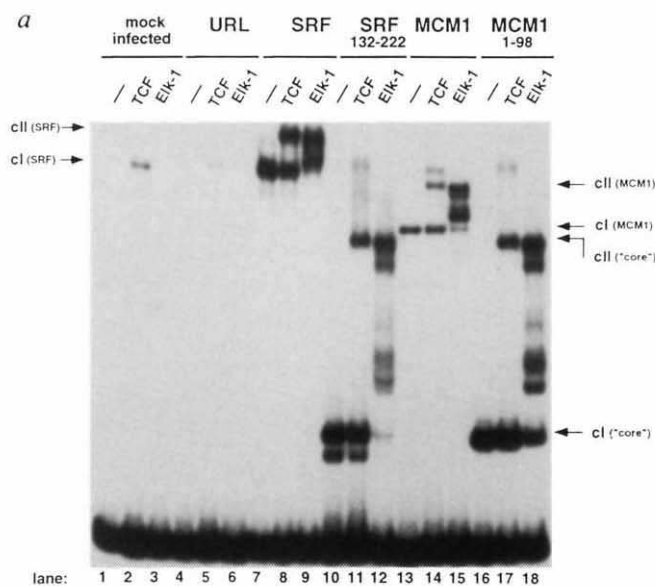


FIG. 2 Elk-1 and p62^{TCF} interact with the same subdomain of SRF and yeast MCM1. *a*, Gel retardation assay of ternary complex formation between Elk-1 or p62^{TCF} and either SRF, MCM1, or the truncation mutants SRF₁₃₂₋₂₂₂ or MCM1₁₋₉₈. Mock infected extract was prepared from cells infected with nonrecombinant vaccinia virus. TCF, URL, cI and cII were described in the legend to Fig. 1. The p62^{TCF} fraction also contains an activity, probably SRF, that generates the additional complex at the position of cI(SRF) in lanes 2, 5, 14 and 17. *b*, Ternary complex formation between Elk-1 or p62^{TCF} and yeast ARG80, SRF₇₀₋₂₄₄, or ARG80 mutants containing SRF-derived amino-acid substitutions¹⁵. *c*, Comparison of SRF with the SRF-homologous yeast proteins MCM1 and ARG80. The boxes represent the portion of the protein remaining in the respective truncation mutants, whose end points are indicated by the amino-acid position. The arrow indicates the homologous core domain. For SSM3 and 4 the black boxes and the numbers in parentheses show the SRF region inserted into ARG80 at the corresponding position in the core domain. SSM3 effectively contains 9 SRF-specific amino acids and SSM4 has three SRF-derived residues¹⁵. In MCM1 the core homology confers an additional specificity to interact with STE12 (ref. 15). METHODS. SRF and MCM1 were generated using recombinant vaccinia virus. SRF₇₀₋₂₄₄, SRF₁₃₂₋₂₂₂, MCM1₁₋₉₈, ARG80, SSM3 and SSM4 have been described^{15,18}. They were synthesized by *in vitro* transcription and translation, and bacterially produced Elk-1 was used in these reactions. The analyses were done as described in the legend to Fig. 1.

		TERNARY COMPLEX FORMATION	
		Elk-1	p62 ^{TCF}
<i>c</i>	SRF	+	+
	SRF ₇₀₋₂₄₄	+	+
	SRF core	+	+
	MCM1	+	+
	MCM1 core	+	+
	ARG80	-	-
	SSM3 (196-210)	+	+
	SSM4 (196-203)	(+)	(+)

ternary complexes with the SRE. Elk-1 and p62^{TCF} have the same DNA sequence requirements and antibodies against Elk-1 block the binding of both proteins. Furthermore, we show that like p62^{TCF}, Elk-1 forms complexes with the yeast SRF-homologue MCM1 but not with yeast ARG80 (ref. 15). But ARG80 mutants that convey interaction with p62^{TCF} can also form complexes with Elk-1. The similarity, or even identity, between Elk-1 and p62^{TCF} suggests a novel regulatory role for Ets proteins that is effected through interaction with other proteins, such as SRF. Furthermore, the possible involvement of an Ets protein in the control of *c-fos* has interesting implications for proto-oncogene cooperation in cellular growth control¹⁶.

The gel retardation assay in Fig. 1 shows the two complexes that form on the *c-fos* SRE in the presence of SRF and p62^{TCF}. p62^{TCF} alone cannot bind to the SRE (lane 1) but forms a ternary complex with it (labelled cII) in the presence of a dimer of either SRF (lane 7) or an internal region, SRF₉₀₋₂₄₄, which includes the domain essential for DNA binding and dimerization^{17,18} (lane 13). *In vivo* observations⁵⁻⁹ indicate that both ternary complex formation and efficient *fos* induction involve a sequence at the 5' border of the SRE that resembles the DNA recognition site for members of the *ets* proto-oncogene family¹¹⁻¹³. Therefore we screened various Ets proteins for p62^{TCF}-like activity. Although neither Ets1 nor Ets2 (ref. 19)

can form ternary complexes (R.A.H. and J. Ghysdael, unpublished observations), the protein encoded by the *Ets*-related gene *elk-1* can interact with SRF. Elk-1 differs from other Ets proteins in having its Ets DNA-binding domain located at the amino terminus of the protein²⁰. We tested Elk-1 generated by *in vitro* transcription/translation or expressed in bacteria. Neither version of Elk-1 binds to the *c-fos* SRE on its own (Fig. 1, lanes 2 and 4), whereas both can form ternary complexes with SRF and SRF₉₀₋₂₄₄ (cII, lanes 8, 10, 14 and 16) that show identical mobility to complexes generated with p62^{TCF}. The bacterial Elk-1 protein generates additional ternary complexes of intermediate mobility, suggestive of some protein degradation, that are not seen with control extracts (lanes 11 and 17). Methylation interference studies establish that they are Elk specific (see legend to Fig. 3).

p62^{TCF} also forms ternary complexes with the yeast transcription factor MCM1 over the *c-fos* SRE (Fig. 2a)¹⁵. MCM1 and SRF are homologous over a core region, namely SRF₁₃₂₋₂₂₂ and MCM1₁₋₉₈, that defines dimerization, specific DNA binding and ternary complex formation (Fig. 2c)^{15,17,18}. They also show functional homology in that both regulate gene expression through multiprotein complexes^{4-9,21}. Elk-1 interacts with MCM1 in a manner similar to p62^{TCF} (Fig. 2a, lanes 14 and 15) and both proteins form ternary complexes with MCM1₁₋₉₈ (lanes

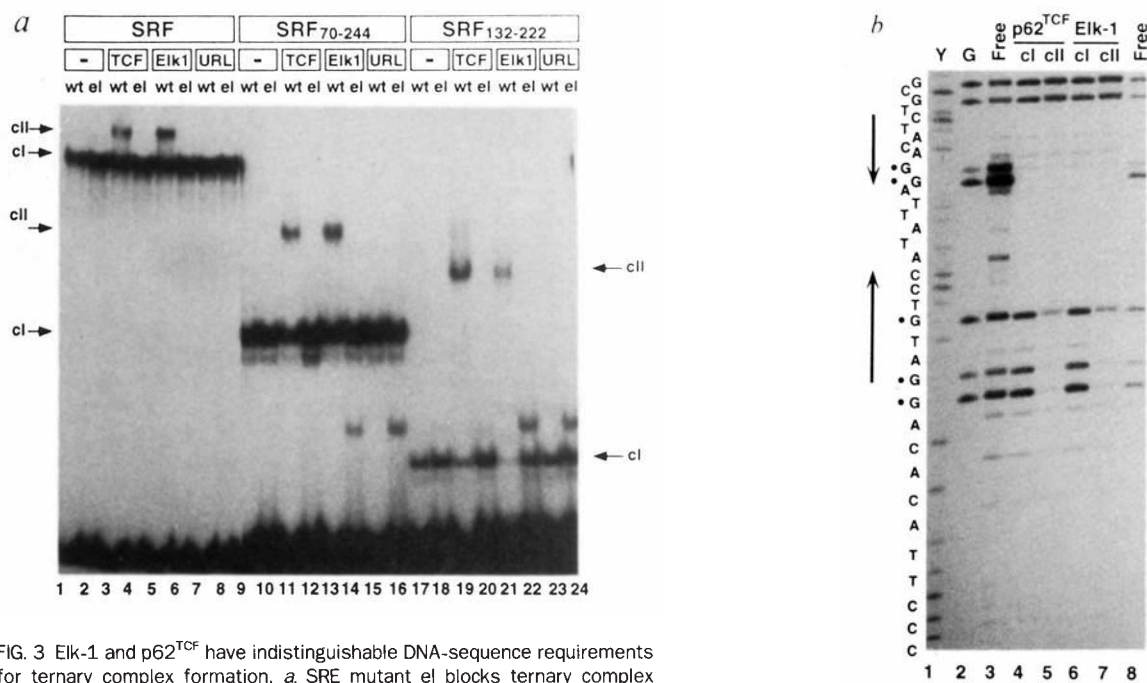


FIG. 3 Elk-1 and p62^{TCF} have indistinguishable DNA-sequence requirements for ternary complex formation. *a*, SRE mutant el blocks ternary complex formation by Elk-1 and p62^{TCF}. Complexes were assembled with either wild-type (wt) SRE probe or the mutant el (see *c*) and the following proteins, as indicated: p62^{TCF}, *in vitro* produced Elk-1 and control reticulocyte lysate (URL) with SRF, SRF₇₀₋₂₄₄ and SRF₁₃₂₋₂₂₂. Binding was assayed as above. A component present in the reticulocyte lysate forms a complex with probe el that migrates slightly slower than SRF₁₃₂₋₂₂₂ cl (lanes 6, 8, 14, 16, 22 and 24). The SRF lanes show a shorter exposure of the same gel. *b*, Methylation interference analysis of the ternary complexes. The upper strand of the SRE is indicated by the sequence beside the autoradiogram. The methylation sensitivity for the ternary complexes (cII) formed by p62^{TCF} (lane 5) and *in vitro* synthesized Elk-1 (lane 7) is shown, along with the corresponding binary complexes (cl) containing SRF alone (lanes 4 and 6), uncomplexed DNA (free, lanes 3 and 8), the input methylated DNA (G, lane 2) and a pyrimidine ladder (Y, lane 1). Arrows indicate the dyad-symmetric SRE, and dots show the sensitive G residues. Identical results have been obtained with SRF₇₀₋₂₄₄, SRF₉₀₋₂₄₄, and SRF₁₃₂₋₂₂₂ and bacterially expressed Elk-1, where the faster ternary complexes also show the same pattern. *c*, Summary of the methylation interference patterns. The upper strand sequence across the *c-fos* SRE is shown, as are the three bases mutated in el. Arrows, G residues whose methylation disrupts complex

formation; smaller arrows, G having an intermediate effect. Diamonds, pattern of methylation protection observed *in vivo*^{5,6}. METHODS. The source of proteins and reaction conditions are described in the other figure legends. Preparative scale binding reactions were done with partially methylated wt SRE probe and processed as described⁷.

17 and 18). As with SRF and SRF₁₃₂₋₂₂₂ (lanes 9 and 12), the bacterially expressed Elk-1 gives rise to additional faster complexes with MCM1 and MCM1₁₋₉₈ (lanes 15 and 18). By contrast, neither p62^{TCF} nor Elk-1 can interact with another yeast SRF-homologue, ARG80, although ARG80 binds to the *c-fos* SRE (Fig. 2b, lanes 4-6)¹⁵. But replacing nine amino acids in ARG80 with those from the corresponding positions in SRF now allows the ARG80 mutant SSM3 to form ternary complexes with p62^{TCF} almost as well as SRF₇₀₋₂₄₄ (Fig. 2b, lanes 11 and 14)¹⁵. An even shorter three amino-acid swap, mutant SSM4, is also weakly active (lane 8). Elk-1 also interacts with these two ARG80 mutants (lanes 9 and 12), and these complexes once again comigrate with those containing p62^{TCF}. As summarized in Fig. 2c, p62^{TCF} and Elk-1 require the same subdomain of SRF and MCM1 for ternary complex formation.

The similarity between p62^{TCF} and Elk-1 also extends to their DNA sequence requirements for ternary complex formation. The mutant 'el' changes the 5' sequence of the SRE from CAGGA to ACTGA (Fig. 3c). This greatly reduces the level of *fos* induction *in vivo*^{7,9} and ternary complex formation *in vitro*⁷. An 'el' probe forms normal binary complexes with SRF and the truncation mutants SRF₇₀₋₂₄₄ and SRF₁₃₂₋₂₂₂ (Fig. 3a, lanes 2, 10 and 18). By contrast, neither p62^{TCF} nor Elk-1 can form ternary complexes with el, although they both interact with the SRF derivatives and wild-type SRE probe (Fig. 3a). Methylation interference studies show more precisely that the p62^{TCF} and Elk-1 ternary complexes are indistinguishable (Fig. 3b). The two adjacent G residues at the 5' boundary of the SRE are clearly underrepresented in their ternary complexes, and the next G residue downstream is also weakened (lanes 5 and 7). These contacts match those observed *in vivo*^{5,6} and are summar-

ized in Fig. 3c. The data demonstrate that Elk-1 and p62^{TCF} are alike in their binding properties.

Finally, we have used specific antisera to probe the interactions involved in ternary complex formation by p62^{TCF} and Elk-1 (Fig. 4). An antiserum against SRF retards the mobility of both the SRF-SRE binary complex and the ternary complex including p62^{TCF} (lane 3), whereas preimmune serum has no effect (lane 2). This is more obvious with SRF₉₀₋₂₄₄ (lane 15). Using this antiserum, a supershift is also observed with the ternary complexes generated with Elk-1 (lanes 9 and 21). Thus, this antiserum against SRF does not block these interactions but instead can still recognize SRF and SRF₉₀₋₂₄₄ present in ternary complexes with Elk-1 and p62^{TCF}.

p62^{TCF} and Elk-1 are antigenically related. An Elk-1-specific antiserum progressively decreases ternary complex formation by Elk-1 and concomitantly increases the amount of the binary complex cI (Fig. 4, lanes 11, 12, 23 and 24). This antiserum has identical effects on the interaction of p62^{TCF} with either SRF or SRF₉₀₋₂₄₄ (lanes 5, 6, 17 and 18), whereas preimmune serum does not (lanes 4 and 16). At lower Elk-1 antiserum concentrations (lanes 5, 11, 17 and 23, data not shown), an additional weak supershift appears with both Elk-1 and p62^{TCF}. This suggests that yet another epitope is shared between these two proteins.

Our data demonstrate that Elk-1 is clearly a ternary complex factor very similar, if not identical, to p62^{TCF}. The ability of Elk-1 and p62^{TCF} to interact with MCM1, together with the functional similarities between MCM1 and SRF^{15,21}, suggests that Elk-1-related Ets proteins may function in yeast and hints at evolutionary conservation of strategies for gene control. The fact that Elk-1 can form complexes with SRF makes it likely that Elk-1 is involved in the multiprotein complex assembled constitutively over the *c-fos* SRE in certain cells⁵⁻⁹. Thus Elk-1 could represent a subclass of Ets proteins that are involved in gene regulation not only by direct DNA binding^{11-13,22-24} but also by cooperation with other factors. Indeed, heteromeric complex formation has also been found with the Ets-related α subunit of the mammalian transcription factor GABP (ref. 25). Furthermore, Elk-1 could also function in the activation of other immediate early genes that contain SREs as key control elements. The cell type- and tissue-specific expression of Elk-1 (ref. 14) may indicate such a regulatory role for this protein during development and differentiation. Consistent with this is the potential relationship between chromosomal rearrangements near the Elk-1 locus and the generation of synovial sarcoma in humans^{14,26}.

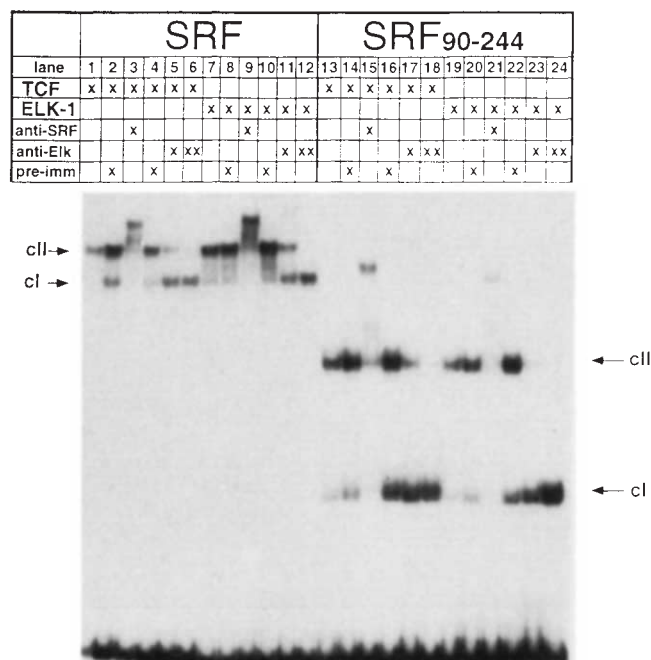


FIG. 4 p62^{TCF} is antigenically related to Elk-1. The components in each binding reaction are indicated above each lane of the gel retardation autoradiogram. Rabbit polyclonal antisera against SRF or Elk-1 and the appropriate preimmune serum was used. Not shown is that the complexes of anti-SRF antibody with SRE:SRF or SRE:SRF₉₀₋₂₄₄ alone migrate slightly slower than the ternary complex cII (lower supershifted band in lanes 3, 9, 15 and 21). The abbreviations are defined in the other figure legends. METHODS. Binding reactions were assembled with either p62^{TCF} or *in vitro* translated Elk-1 and the appropriate antiserum. After incubation for 30 min at room temperature, SRF or SRF₉₀₋₂₄₄ was added and the incubation continued, after which the complexes were separated as above. The order of addition of the antisera makes no difference in the results obtained, nor does using *in vitro* translated SRF₇₀₋₂₄₄ or SRF₁₃₂₋₂₂₂.

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